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### Process and apparatus for dyeing textiles

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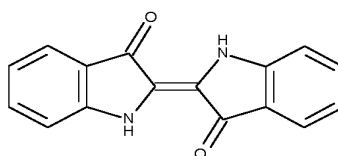
## PROCESS AND APPARATUS FOR DYEING TEXTILES

This invention relates to a process and apparatus for dyeing of textiles, as well as to an immobilized enzyme comprised in said apparatus required for carrying out the process.

### BACKGROUND OF THE INVENTION

Vat dyes are insoluble dyes that require a reducing agent to be solubilized in water. Conventionally, dyeing with vat dyes includes applying the dye in its soluble, reduced form to the textiles and subsequently oxidizing the dye back to the insoluble form, which confers color to the textile.

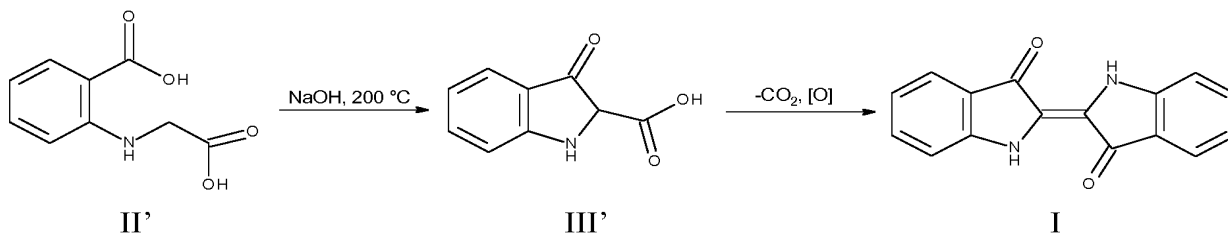
Indigo is a vat dye of Formula I:



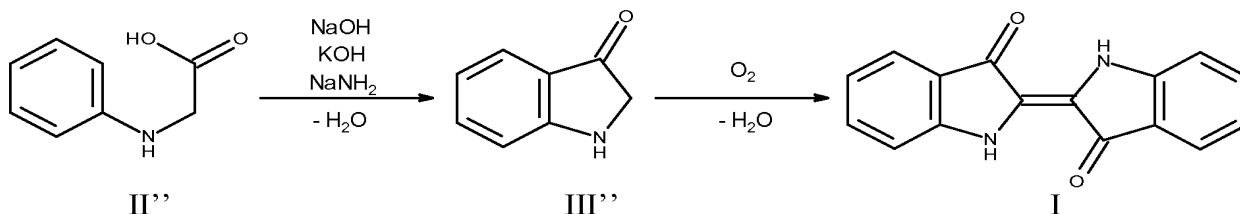
Formula I

Substitutions on the indigo aromatic ring(s) with groups such as halogen, alkyl, alkoxy, amino, aryl, aryloxy, and carbonyl, provide compounds that span in a wide range of colors other than blue, and are part of the so-called indigo derivatives.

A large percentage of indigo and indigo derivatives is produced via synthesis. Heumann synthesis (Scheme 1 below) and Pfleger synthesis (Scheme 2 below) were the first synthetic routes used for indigo manufacturing at industrial scale; variations of these methods are still in use today.



Scheme 1

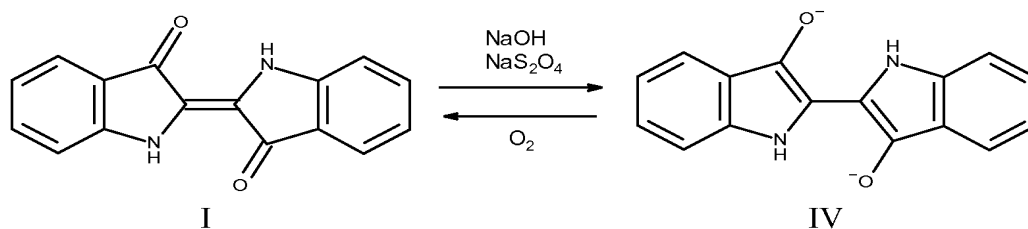


Scheme 2

The above synthetic routes are also used to manufacture indigo derivatives; in this case, compounds II', II'', III' and III'' are substituted in the same position(s) with the same group(s) as in the desired indigo derivative to be synthesized.

Synthesis of indigo and derivatives thereof, as well as other vat dyes, can also be carried out by means of enzymes, or by bacteria expressing enzymes. However, such enzymatic synthesis are not employed in industrial processes.

While precursors of indigo (such as compounds II', II'', III' and III'' of Schemes 1 and 2 above) are soluble in aqueous solutions, indigo is not, and it precipitates after its synthesis in aqueous solutions. Therefore, as said above, indigo or derivatives thereof have to be reduced (by treatment with reducing agents, for example as shown in Scheme 3).



Scheme 3

wherein compound I is indigo, and compound IV is the water soluble and reduced form of indigo, called leuco-indigo (or indigo white, as it is colorless).

Therefore, industrial dyeing methods using indigo or derivatives thereof as a dye, or using vat dyes in general, comprise first the synthesis in aqueous solution of indigo or derivatives thereof (or of vat dyes) in reactors through known methods (*e.g.* the synthetic route of Scheme 1 above). Indigo or derivatives thereof (or vat dyes) are obtained as a precipitate. The aqueous solution comprising suspended indigo or derivatives thereof (or the vat dyes) is then treated with reducing agents to obtain an aqueous solution comprising dissolved leuco-indigo or derivatives thereof (or reduced-vat dyes). The aqueous solution comprising dissolved leuco-indigo or derivatives thereof (or reduced-vat dyes) is then applied onto textiles. Once the solution comprising dissolved leuco-indigo wets the textiles, indigo or derivatives thereof (or the vat dyes) are obtained by oxidation of leuco-indigo or derivatives thereof (or leuco-vat dye), and the textiles are thus dyed. Such oxidation can be carried out, for example, with the oxygen in the air. Usually, the indigo dyeing process requires several impregnation and oxidation steps to reach the desired shade of colour.

The reducing agents used to reduce insoluble vat dyes, such as indigo or derivatives thereof, are harsh chemicals, *i.e.* hazardous chemicals for users and/or environment, such as sodium hydroxide

and sodium hydrosulfite. Indeed, large quantities of reducing salts and hydroxides are used in conventional dyeing processes wherein, for example, indigo or derivatives thereof are used as dyes, thus generating great amounts of wastewater that must be treated before being disposed. This step adds to costs of the dyeing process.

5 There is thus a need for an improved method for dyeing textiles with vat dyes, in particular with indigo and derivatives thereof, that reduces the cost for vat dyeing and of the water treatment processes.

A further problem with known indigo dyeing process is that the textile, especially cellulose, may be damaged by extended exposure to the alkaline process solution and to the by-product therein  
10 present.

#### SUMMARY OF THE INVENTION

Aim of the present invention is to solve the above problem and to provide a process for dyeing textiles using insoluble dyes, such as vat dyes, in particular using indigo and derivatives thereof, that is safe, cost-effective and environmentally friendly.

15 Another aim of the present invention is to provide a process for dyeing textiles using indigo and derivatives thereof, as well as insoluble dyes, such as vat dyes, that is more sustainable with respect to conventional dyeing methods using indigo and derivatives thereof, or vat dyes in general.

The above aims, as well as others, are reached through the present invention that provides a process according to claim 1, namely a process for dyeing a textile, comprising an enzymatic synthesis of  
20 a dye precursor, characterized in that it comprises the following steps:

- a) contacting a solution comprising at least a first dye precursor with at least a first immobilized enzyme, to convert at least part of said first dye precursor into at least a second dye precursor, to obtain a solution comprising said second dye precursor;
- b) generating a flow of the solution comprising the second dye precursor, whereby the solution  
25 comprising said second dye precursor flows from the first immobilized enzyme to the textile;
- c) contacting the solution comprising the second dye precursor with a textile; and
- d) converting at least part of the second dye precursor to at least one dye, whereby at least part of the textile is dyed;

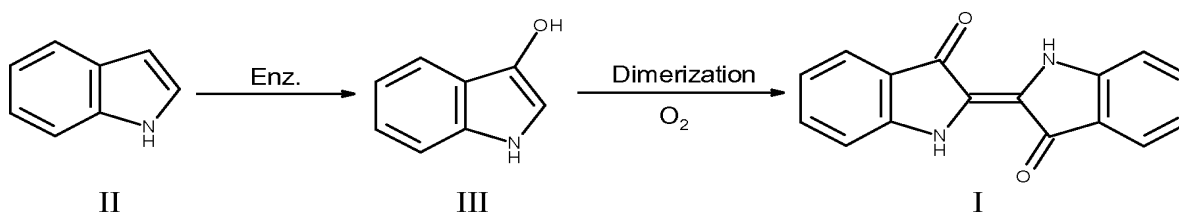
wherein said first immobilized enzyme is spaced apart from the textile.

30 In the following description, “textile” or “textiles” refer to any fibers, yarns, ropes, fabrics and/or garments able to be dyed, for example by indigo and/or derivatives thereof. The textile materials

may be of natural origin, such as the ones deriving from animals or plants, *e.g.* cotton, linen, silk, wool, etc., or may be of synthetic origin, or may be mixtures thereof, such as elasticized cotton fabrics or garments. Moreover, said yarns may be manufactured by any known method, and said fabrics also may be manufactured by any known method, such as weaving, knitting, crocheting, knotting, and felting. Furthermore, said garments may be any garment, such as jeans, shirts, casual wear garments, etc.

In the present invention, “first dye precursor” refers to any soluble compound that can be converted enzymatically into a second dye precursor. In the present invention, “second dye precursor” refers to any soluble compound that can be obtained by a first dye precursor and can be converted to an insoluble dye, for example via dimerization. In the present invention, “insoluble dye” refers to any water-insoluble compound that is conventionally used to dye textiles, for example any vat dye, such as indigo. Therefore, according to the present invention, first dye precursor, second dye precursor and insoluble dye are related to each other via a synthetic route, in particular a synthetic route comprising a first enzymatic step and a second non-enzymatic step. For example, said first dye precursor, said second dye precursor and said insoluble dye according to the present invention can be, respectively, indole and/or derivatives thereof, indoxyl and/or derivatives thereof, and indigo and/or derivatives thereof, and are related as represented in Scheme 4 reported below. Which first dye precursor has to be comprised in the solution of step a) is chosen according to the color the textiles to be dyed must have at the end of the dyeing process of the invention, as the color of the textiles will be provided by the insoluble dye and the insoluble dye is obtained starting from the first dye precursor.

Advantageous examples of first dye precursor, second dye precursor, and insoluble dye are, respectively, indole and/or derivatives thereof, indoxyl and/or derivatives thereof, and indigo and/or derivatives thereof. Indigo and its derivatives can be synthesized starting from indole and its derivatives thereof, as represented in Scheme 4 below.



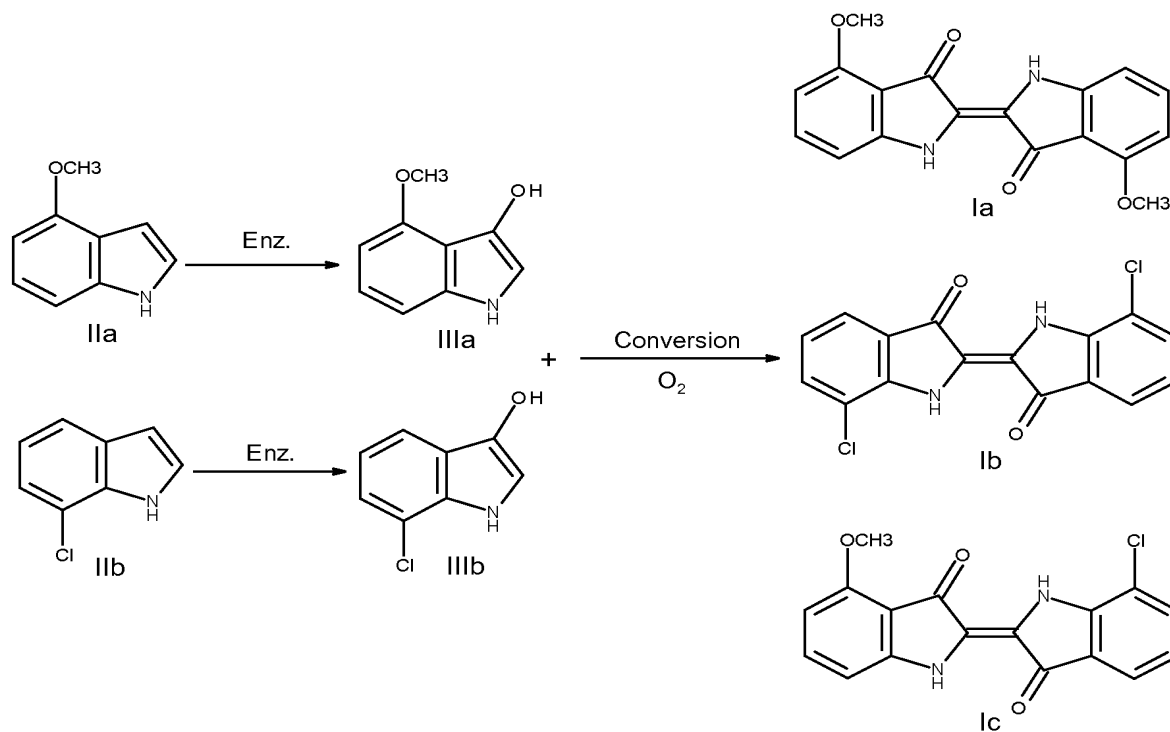
Scheme 4

wherein compound II is indole (first dye precursor), compound III is indoxyl (second dye precursor), and compound I is indigo (the dye). In particular, with reference to Scheme 4 reaction, the addition of a hydroxyl group to the carbon in position 3 of indole provides indoxyl, which dimerize into indigo when in aqueous solution. According to the present invention, the addition of a hydroxyl group to indole and/or derivatives thereof is carried out by the first immobilized enzymes in step a) of the process of the invention. When the first dye precursor is indole and/or derivatives thereof, the second dye precursor is indoxyl and/or derivatives thereof, and the insoluble dye is indigo and/or derivatives thereof, the process of the invention results in dyeing textiles with indigo and/or derivatives thereof, which are ones of the most used dyes in textile dyeing, without passing through the reduction steps of the prior art.

According to the present invention, “indole derivatives”, “indoxyl derivatives” and “indigo derivatives” refer to respectively indole, indoxyl and indigo substituted by one or more substituents, for example substituted by: one or more groups on one or more carbons in any position selected from positions 4, 5, 6 and 7 of indole or indoxyl, and from positions 4, 4', 5, 5', 6, 6', 7, and 7' of indigo, and/or by a group on the nitrogen atom(s) of indole, indoxyl or indigo. The one or more groups substituting one or more carbons may be groups such as, but not limited to, halogen groups, alkyl groups (*e.g.* C<sub>1</sub>-C<sub>20</sub> alkyl groups, such as methyl, ethyl, propyl, isopropyl, butyl, tert-butyl, and iso-butyl), alkoxy groups (*e.g.* C<sub>1</sub>-C<sub>20</sub> alkoxy groups, such as methoxy, ethoxy, butoxy, tert-butoxy, and iso-butoxy), aryl groups (*e.g.* phenyl, substituted phenyl, benzyl, substituted benzyl, naphthyl, anthracenyl, and heteroaryl), aryloxy groups (*e.g.* phenoxy, and naphyloxy), amine groups (*e.g.* primary and/or secondary aliphatic and/or aromatic amine groups), nitro groups and carbonyl groups (*e.g.* aldehyde groups, such as aromatic and/or aliphatic aldehyde, and ketones, such as aromatic and/or aliphatic ketones). The group substituting nitrogen atom(s) may be groups such as, but not limited to, alkyl groups (*e.g.* C<sub>1</sub>-C<sub>20</sub> alkyl groups, such as methyl, ethyl, propyl, isopropyl, butyl, tert-butyl, and iso-butyl), aryl groups (*e.g.* phenyl, substituted phenyl, benzyl, substituted benzyl, naphthyl, anthracenyl, and heteroaryl), and acyl groups (such as formyl, acetyl, propionyl, benzoyl, and acryloyl). Therefore, indole derivatives may be, for example, 4-chloroindole, 5-chloroindole, 6-chloroindole, 7-chloroindole, 5-bromoindole, 6-bromoindole, 5-nitroindole, 5-hydroxyindole, 5-methylindole, 5-methoxyindole, 6-methylindole, 7-methylindole, 5-aminoindole, 1-methylindole, indole-6-carboxaldehyde; and indoxyl derivatives can be, for example, 4-chloroindoxyl, 5-chloroindoxyl, 6-chloroindoxyl, 7-chloroindoxyl, 5-bromoindoxyl, 6-bromoindoxyl, 5-nitroindoxyl, 5-hydroxyindoxyl, 5-methylindoxyl, 5-methoxyindoxyl, 6-methylindoxyl, 7-methylindoxyl, 5-aminoindoxyl, 1-



methylindoxyl, indoxyl-6-carboxaldehyde. It is encompassed in the present invention also the use of any other indole and indoxyl derivatives, provided that such indole derivatives can be reacted and converted into the correspondent indoxyl derivatives by enzymatic catalysis. These indoxyl derivatives, when converted (*i.e.* dimerized according to step d) of the process of the invention), provide the correspondent indigo derivatives, which have each a different color. According to the present invention, “indigo derivatives” refer also to asymmetric indigo, *i.e.* indigo deriving from dimerization of two different indoxyl derivatives, or of indoxyl and an indoxyl derivative. Dyeing of the textile with asymmetric indigo can be achieved according to the process of the invention when a solution comprising two or more different indole derivatives, or indole and one or more indole derivatives, is contacted with the enzyme in step a). For example, when two different indole derivatives, or indole and an indole derivative, are contacted with the first immobilized enzyme (step a)), two different indoxyl derivatives, or indoxyl and an indoxyl derivative, are obtained; which are subsequently contacted to the textile according to step c) of the process. When such two different indoxyl derivatives, or indoxyl and an indoxyl derivative, convert to at least a dye according to step d) of the process, three different indigo derivatives are obtained, namely two different symmetric indigo derivatives and an asymmetric indigo derivative (for example, as represented in Scheme 5 below), and the textile is thus dyed with more than one dye, one of which is asymmetric indigo.



Scheme 5

With reference to Scheme 5, the two different indole derivatives are compounds IIa (4-methoxyindole) and IIb (7-chloroindole), the two different indoxyl derivatives are compounds IIIa (4-methoxyindoxyl) and IIIb (7-chloroindoxyl), and the three different indigo derivatives are compounds Ia (4,4'-dimethoxyindigo or 4-methoxy-2-(4-methoxy-3-oxo-1,3-dihydro-2H-indol-2-ylidene)-1,2-dihydro-3H-indol-3-one; symmetric indigo derivative), Ib (7,7'-dichloroindigo or 7-chloro-2-(7-chloro-3-oxo-1,3-dihydro-2H-indol-2-ylidene)-1,2-dihydro-3H-indol-3-one; symmetric indigo derivative), and Ic (7-chloro-4'-methoxyindigo or 7-chloro-2-(4-methoxy-1,3-dihydro-3-oxo-2H-indol-2-ylidene)-1,2-dihydro-3H-indol-3-one; asymmetric indigo derivative). Therefore, when at least two indole derivatives, or indole and at least one indole derivative, are contacted with the enzyme, more than one indigo derivatives can be obtained in step d) of the process, whereby dyeing of textile with more than one dye is achieved according to the process of the invention.

According to the present invention, "first immobilized enzymes" refer to any enzymes able to catalyze the conversion of a first dye precursor to a second dye precursor according to step a) of the process of the invention. For example, the first immobilized enzyme can be an immobilized enzyme that is able to catalyze the conversion (*i.e.* oxidation) of indole and/or derivatives thereof to indoxyl and/or derivatives thereof.

According to the present invention, contacting any compound to enzymes, such as the first dye precursor to the immobilized enzyme in step a), means allowing such compound to be converted enzymatically. This contacting can be carried out by contacting a flow of the solution comprising at least such first dye precursor to the enzyme, whereby the dissolved first dye precursor comprised in such solution contacts in turn the immobilized enzyme and is converted enzymatically to the second dye precursor.

According to the present invention, contacting the solution comprising at least the second dye precursor with a textile, means wetting the textile with such solution, whereby the textile is impregnated with such solution in which the second dye precursor is dissolved.

According to the present invention, "immobilized" or "immobilization" refer to the immobilization process of enzymes. Immobilization of enzymes is a conventional process known in the art, which involves the attachment, preferably covalent attachment, of such enzymes to carriers, for example to epoxy-activated resins (such as methacrylate copolymers, *e.g.* Eupergit®, SepaBeads®, Relizyme™, Purolite®), cellulose, agarose, polystyrenic ion exchange resins, amino acrylate resins, hydrogels (immobilization by occlusion; *e.g.* agarose, alginate, carrageenan or gelatin),

chelating carriers (*e.g.* Ni-Sepharose®, IDA-Sepharose®, NTA-Sepharose®, IDA-Agarose and derivatives of), etc. The type of carriers used to immobilize enzymes might depend on which are the exposed groups of the enzymes. For example, if surface amino groups are exposed on the enzymes, epoxy-activated resins may be used as carriers: as the amino groups covalently attaches  
5 to the epoxy groups of the epoxy-activated resins, the enzymes are immobilized onto the epoxy-activated resins. According to the invention, immobilization may be carried out by incubating the enzymes and epoxy-activated resins with agitation for 12 h in a 100 mM potassium-phosphate solution of pH 8.0 with 0.5 M NaCl. Advantageously, the remaining (unreacted) activated groups of the carriers are inactivated after immobilization has been carried out; for example, inactivation  
10 of the remaining activated groups is carried out by incubating 10 mM ethanolamine or 10 mM glycine in the solution comprising epoxy-activated resins.

Suitable epoxy-activated resins have particle size comprised in the range from 100 to 1100 micron, *e.g.* from 150 to 300 micron, or from 200 to 500 micron, or from 250 to 1000 microns, with mean pore diameter comprised in the range from 300 to 1800 angstroms, for example from 300 to 600  
15 angstroms, or 1200 to 1800 angstroms. In an aspect of the invention, immobilization of enzymes can be carried out in a ratio comprised in the range of 5-75 mg of (semi)purified enzymes per gram of wet carrier, more preferably in a ratio comprised in the range of 15-25 mg of (semi)purified enzymes per gram of the wet carrier.

Mutant enzymes (*e.g.* genetically modified enzymes) can be employed in the process of the  
20 invention, for example in order to increase the catalyzing efficiency or to provide an improved binding to carriers. For example, when the carriers used to immobilize the enzymes are epoxy-activated resins, the enzymes can be modified with the introduction of a hexalysine (6xLys) or a hexahistidine (6xHis) tag sequence, preferably on the N-terminus, to increase their binding to carriers.

According to the present invention, “spaced apart” refers to spacing the first immobilized enzyme and the textile so that conversion of the second dye precursor to the insoluble dye does not occur at and/or near said first immobilized enzyme, and occurs instead after the solution comprising the second dye precursor is contacted with the textile. Therefore, conversion of the second dye precursor to the insoluble dye is obtained directly onto the textiles. According to the invention, the  
25 textiles are thus located downstream of the immobilized enzyme with respect to the direction of the flow of the solution. The enzymes are immobilized so that they are confined and cannot flow along with the flow of the solution. Such spacing can be carried out, for example, by separating the immobilized enzyme (or enzyme system) and textile in different containers or chambers, or by  
30

confining the immobilized enzyme (or enzyme system) and said textile in different areas of the same container or chamber. Parameters of the solution, such as flow rate, temperature, and contact time of the solution with the enzyme and the textile, may be selected to ensure that conversion of the second dye precursor to the insoluble dye occurs substantially on the textile, *i.e.* after the solution comprising at least the second dye precursor is contacted with the textile.

Therefore, according to the present invention, conversion (*i.e.* precipitation) of the second dye precursor to the insoluble dye at and/or near said first immobilized enzyme is substantially prevented. It was found by the applicant that precipitation of insoluble dye at and/or near the enzymes may result on a loss of enzyme activity; it is deemed that the precipitated insoluble dye negatively affects the substrate-enzyme interaction, preventing further contact of the substrate (*i.e.* the first dye precursor) with the immobilized enzyme.

Another advantage of the invention is that the use of reducing agents is avoided; therefore, the process of the invention is environmentally friendly, safe, cost-effective and sustainable, as it does not produce wastewaters containing reducing agents.

When the process is carried out in standard dyeing conditions and the second dye precursors are indoxyl and/or derivatives thereof, their dimerization to indigo and/or derivatives thereof is spontaneous. Such spontaneous dimerization, according to the process of the invention, occurs after the solution comprising at least said indoxyl and/or derivatives thereof is contacted to the textile, which is spaced apart from the first immobilized enzyme. The process of the invention allows using enzymatic synthesis of indigo and/or derivatives thereof for dyeing textiles in industrial scale processes.

Advantageously, the process of the invention can be a continuous process. A continuous process may involve adding the first dye precursor upstream of step a) so that a solution comprising the dye precursor is contacted with the immobilized enzyme in continuous.

According to an embodiment of the process of the invention, the solution obtained after step d) is an exhaust solution that may comprise unreacted first dye precursor, which has not been converted to the second dye precursor in step a).

According to an embodiment of the process of the invention, at least part of the exhaust solution is directed back to the first immobilized enzyme after step d) to be recycled into the process. This embodiment allows converting unreacted first dye precursor that is present in the exhaust solution into the second dye precursor, which can then be subjected to steps b) to d) of the process of the invention. The present embodiment optimizes the use of said first dye precursor, as it can be

converted entirely to said second dye precursor, for example by repeating the steps of the process, namely by directing the flow of the solution to contact the first immobilized enzymes several times. Repeating the steps of the process may provide different shades of color to the dyed textile; the process can thus be carried out until the desired shade of color is obtained for the textile.

5 Moreover, this embodiment allows avoiding the dispose of the exhaust solution that may still contain useful solutes, such as unreacted first dye precursor, buffer, co-factors, etc.

Moreover, first dye precursor can be added to the exhaust solution directed back to the immobilized enzyme after step d). This allows carrying out the process of the invention in continuous.

10 In the following paragraph, the process will be disclosed with reference to an embodiment where the insoluble dye is indigo, the second dye precursor is indoxyl, and the first dye precursor is indole. The scope of the invention is not limited to these exemplified compounds. According to an embodiment of the invention, said first immobilized enzyme is an oxidizing enzyme whereby indole and/or derivatives thereof are oxidized to obtain indoxyl and/or derivatives thereof when  
15 contacted by the oxidizing enzyme.

According to the present invention, "oxidizing enzymes" refers to any enzyme that is able to catalyze oxidation of its substrates, such as an oxidoreductase (EC 1). Suitable oxidoreductase is a monooxygenase (EC 1.13); it preferably is a flavin-containing monooxygenase (FMOs) (EC 1.14.13.8), and more preferably a microbial flavin-containing monooxygenase (mFMO).

20 Alternatively, the monooxygenase can be a Baeyer-Villiger monooxygenase (BVMO). Monooxygenases, in particular FMOs and mFMOs, provide good conversion rates and binding of many first dye precursor, such as indole and/or many derivatives thereof, as well as a suitable specificity to convert most indole derivatives, and are thus beneficial for the invention. Baeyer-Villiger monooxygenases (BVMOs) have close homology to FMOs, and are thus beneficial as  
25 well for the invention. A particularly preferred oxidizing enzyme to be used in the invention is mFMO from *Methylophaga sp.*, more preferably from the strain SK1. This type of mFMO is very soluble in aqueous solutions and can provide high concentration solutions, thus increasing the amount of second dye precursor 113, such as indoxyl and/or derivatives thereof, that can be synthesized by means of such type of mFMO. Moreover, this type of mFMO is able to convert  
30 most indole derivatives to the correspondent indoxyl derivative, as it is not specific just to indole or to one particular indole derivative. However, it is also encompassed in the scope of the invention any homologue of said *Methylophaga sp.* mFMO. Genetically modified enzymes, such as

genetically modified oxidizing enzymes, can be used according to the present invention, *e.g.* to improve oxidation efficiency of the first dye precursor.

According to an embodiment of the invention, the process comprises using also a second immobilized enzyme, preferably an immobilized cofactor-regenerating enzyme.

5 According to the invention, the “second immobilized enzyme” may be any enzyme that is able to assist and/or complete the conversion of the first dye precursor to the second dye precursor catalyzed by the first immobilized enzyme. In particular, the second immobilized enzyme can assist such conversion by regenerating cofactor(s) used by the first immobilized enzyme; in such case, the second immobilized enzyme is an immobilized cofactor-regenerating enzyme.

10 According to the present invention, “cofactor-regenerating enzyme” is any enzyme that is able to regenerate (*i.e.* produce) cofactor(s) used by the first immobilized enzyme to catalyze the conversion reaction of the first dye precursor (step a) of the process of the invention).

The second immobilized enzyme may thus produce cofactor(s) used by the first immobilized enzyme starting from a substrate that is less expensive than the cofactor(s) used by said first  
15 immobilized enzyme.

Suitable cofactor-regenerating enzymes are known in the art and are *e.g.* a dehydrogenase, such as a glucose dehydrogenase (GDH), a phosphite dehydrogenase (PTDH), a formate dehydrogenase (FDH), and mutant thereof. Preferably, the cofactor-regenerating enzyme is a phosphite dehydrogenase (PTDH); an example of suitable PTDH is disclosed in WO 2004/108912 A2. Using  
20 dehydrogenases, such as GDH, FDH and in particular PTDH, is advantageous when an oxidizing enzyme, and in particular a FMO, is used as said first immobilized enzyme. GDH, FDH, and PTDH regenerate the cofactor(s) used by such oxidizing enzymes starting from, respectively, glucose, formate, and phosphite salts, which are significantly less expensive than the cofactor(s) used by said oxidizing enzymes, and widely available commercially. Genetically modified cofactor-  
25 regenerating enzymes can be used according to the present invention *e.g.* to improve cofactor regeneration efficiency of the first dye precursor.

Preferably, first and second enzymes are both immobilized to provide an enzymatic system.

According to an embodiment of the invention, the immobilized enzyme system is spaced apart from the textile and the solution flows from the enzymatic system o the textile. The invention  
30 process avoids or at least greatly reduces the precipitation of insoluble dye also at or near the immobilized enzyme system.

According to a further embodiment of the invention, the first immobilized enzyme and the second immobilized enzyme are provided as an immobilized fusion enzyme, and thus a fusion system is provided. Preferably, said first immobilized enzyme is an oxidizing enzyme, more preferably a monooxygenase, even more preferably a microbial flavin-containing monooxygenase (mFMO).

5 Said second enzyme is preferably a cofactor-regenerating enzyme, more preferably a dehydrogenase, even more preferably is at least one dehydrogenase selected from the group consisting of: glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH), and most preferably is a phosphite dehydrogenase (PTDH). Thus, according to a preferred embodiment, said fusion enzyme is PTDH-mFMO fusion enzyme.

10 Fusion of enzymes is a technique known in the art. Suitable fusion enzymes comprise a region deriving from the first enzyme and a region deriving from the second enzyme, each of the region providing the required functional properties.

The immobilization of the fusion enzymes can be carried out by means of surface-exposed groups on any region of the fusion enzymes, *e.g.* surface-exposed groups on the first enzyme region, or  
15 on the second enzyme region, or on both regions. The carriers can be chosen accordingly to the surface-exposed groups on any region of the fusion enzymes.

Mutant fusion enzymes can be employed in the process of the invention. Such mutant fusion enzymes are genetically modified in order *e.g.* to be immobilized more effectively or to improve the catalyzing efficiency.

20 In an embodiment, the first immobilized enzyme, or the enzyme system, is located in a first container, or chamber, and the textile is located in a second container, or chamber. Therefore, according to this embodiment, step a) is carried out in at least a first chamber, and the dyeing of at least part of said textile is carried out in at least a second chamber. Therefore, according to the said embodiment, conversion of the first dye precursor to the second dye precursor (step a) of the  
25 process of the invention) occurs in the first chamber, while conversion of said second dye precursor to said dye occurs in said second chamber, which is in fluid connection with said second chamber.

According to an embodiment, the first dye precursor can be produced enzymatically starting from one or more starting compounds. For example, indole (*i.e.*, an exemplary first dye precursor) can be obtained enzymatically starting from tryptophan as starting compound.

30 According to embodiments, the first dye precursor is produced enzymatically starting from one or more starting compounds in one or more reactors different from the first and second chamber.

Advantageously, according to embodiments, such reactors may be in fluid connection with at least the first chamber.

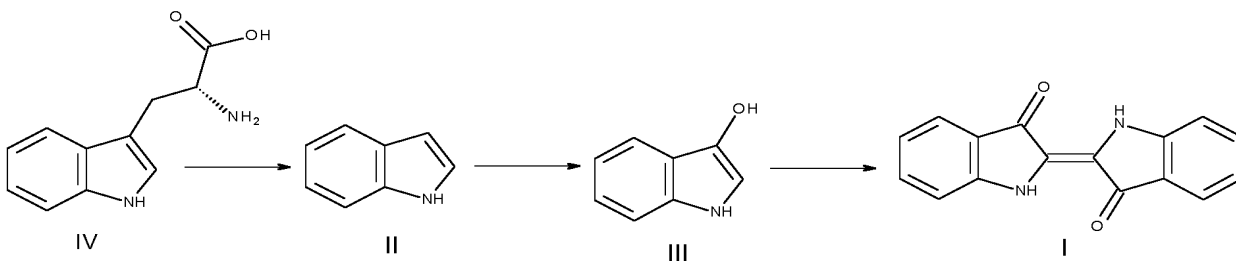
According to embodiments, the first dye precursor is produced enzymatically starting from one or more starting compounds in the first chamber, above mentioned.

5 As used herein, the term “starting compound” refers to a compound that can be converted into a first dye precursor, as defined above, by one or more enzymatic reactions. Such enzymatic reactions are carried out by one or more starting enzymes. According to embodiments, the starting enzymes may be immobilized.

10 According to embodiments, a starting compound (e.g. tryptophan) may be enzymatically converted into a derivative thereof, e.g. a halogenated derivative.

As used herein, the term “starting enzyme” refers thus to one or more enzymes that can catalyze the conversion of a starting compound, e.g., tryptophan, to a first dye precursor, e.g. indole.

Scheme 6 below represents an illustrative reaction scheme wherein a first dye precursor (indole (II)) is obtained enzymatically starting from a starting compound (tryptophan (IV)):



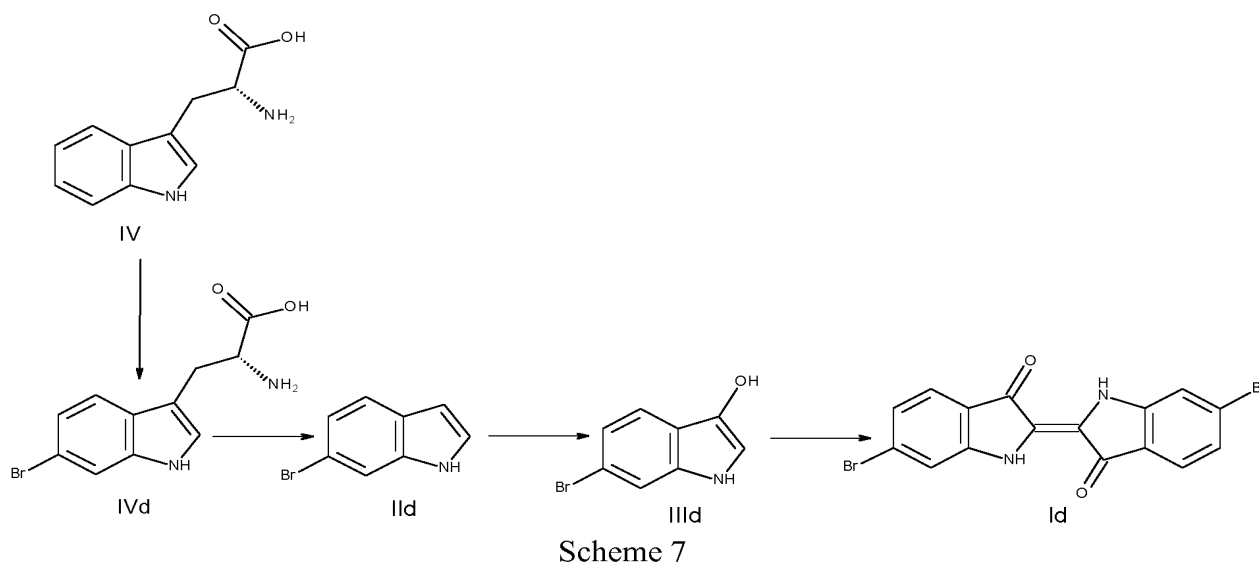
Scheme 6

With reference to Scheme 6, compound IV is tryptophan (starting compound), compound II is indole (first dye precursor), compound III is indoxyl (second dye precursor) and compound I is indigo (insoluble dye). According to embodiments, the conversion of tryptophan to indole can be carried out by a starting enzyme such as tryptophanase or by a mutant form thereof (e.g. a mutant form having improved catalytic properties), while the conversions from first dye precursor to second dye precursor, and from second dye precursor to insoluble dye can be carried out as described above. The reaction of Scheme 6 can also be applied to tryptophan, indole, indoxyl and indigo derivatives.

25 As used herein, the term “Tryptophan derivative” refers to tryptophan substituted by one or more substituents, as above disclosed with reference to indole, indoxyl and indigo derivatives. For example, an illustrative reaction involving a tryptophan derivative, and the correspondent indole,



indoxyl and indigo derivatives, is represented in Scheme 7. Scheme 7 represents an illustrative reaction wherein a starting compound (tryptophan (IV)) is converted into a halogenated derivative (6-bromotryptophan (IVd)). In this case, more than one starting enzymes are required to obtain the desired first dye precursor (6-bromoindole (IId)), i.e., a tryptophan halogenase and a tryptophanase.



With reference to Scheme 7, compound IV is tryptophan (the starting compound), compound IVd is 6-bromotryptophan (halogenated derivative of the starting compound), compound IId is 6-bromoindole (first dye precursor), compound IIId is 6-bromoindoxyl (second dye precursor) and compound Id is 6,6'-dibromoindigo (also called Tyrian purple; insoluble dye). As above mentioned, the conversion of tryptophan to 6-bromotryptophan and conversion of 6-bromotryptophan to 6-bromoindole can be carried out by starting enzymes such as, respectively, tryptophan halogenase and tryptophanase, while the conversions from first dye precursor to second dye precursor, and from second dye precursor to insoluble dye can be carried out as described above.

According to embodiments, the insoluble dye is obtained by means of enzymatic cascade reaction steps (*i.e.* the enzymatic conversions that leads to the second dye precursor starting to the starting compound), followed by a non-enzymatic reaction step (*i.e.* the conversion from the second dye precursor to insoluble dye that occurs onto the textile).

Advantageously, by controlling the parameters of the process of the invention it is possible to obtain the conversion of the second dye precursor to insoluble dye directly onto the textile and to substantially avoid the precipitation of the insoluble dye at and/or near the enzymes.

According to embodiments, the process of the invention may further comprise the steps of contacting a solution comprising at least a starting compound with at least a starting enzyme, to convert at least part of such starting compound into a first dye precursor to obtain a solution comprising at least said first dye precursor, before that step a) is carried out.

A further object of the present invention is an apparatus according to claim 11 to carry out a process for dyeing textiles, namely an apparatus for dyeing a textile comprising a first chamber containing at least a first immobilized enzyme and a solution comprising at least a dye precursor, at least a second chamber containing a textile, and means to generate a flow of a solution. The first chamber is in fluid connection to the second chamber, whereby the solution comprising at least a dye precursor can flow from the first chamber to the second chamber, where at least part of the dye precursor is converted to a dye to dye at least part of the textile. The second chamber optionally comprises outlet means to remove said solution from said second chamber.

According to a further embodiment of the invention, the apparatus further comprises:

- one or more reservoirs in fluid connection with at least the first chamber, so that a solution comprising a dye precursor can flow from the reservoir to the first chamber; and/or
- one or more collection tanks in fluid connection with the outlet means of the second chamber.

The reservoir according to the present embodiment can be set to provide a solution comprising at least a first dye precursor, such as the first dye precursor, to the apparatus of the invention, and in particular to the first chamber containing the first immobilized enzymes. Indeed, said reservoir can be set on purpose so that a user can feed easily solutions and/or solutes to the apparatus of the invention, such solutions and/or solutes being required to carry out the process of the invention by means of the apparatus of the invention. Therefore, solutions, for example comprising at least said first dye precursor, can be added to said reservoir, and such solution can then be fed to said first chamber containing said first immobilized enzymes (and eventually said second enzymes) by means of the fluid connection between said reservoir and said first chamber. The collection tank according to the present embodiment allows collecting the exhaust solution that is obtained once the textiles are dyed (such as after step d) of the process of the invention).

According to a further embodiment, the apparatus of the invention also comprises means to generate a flow of a solution. Said means to generate a flow of a solution, such as one or more pumps, allows the flow of solutions comprised in the apparatus of the invention.

According to embodiments, the apparatus may comprise also one or more reactors in fluid connection with the apparatus, preferably with the first chamber. Said one or more reactors may contain one or more starting enzymes and a solution comprising one or more starting compound.

According to embodiments, one or more starting enzymes and a solution comprising one or more starting compound may be contained in the first chamber to enzymatically produce the first dye precursor.

A further object of the present invention is an immobilized fusion enzyme according to claim 19, namely an immobilized fusion enzyme comprising a carrier and at least a fusion enzyme immobilized on said carrier, wherein said fusion enzyme is the PTDH-mFMO fusion enzyme.

The PTDH-mFMO fusion enzyme was found to be as particularly useful for carrying out the process of the invention, *i.e.* dyeing of textiles, in particular when said dyeing has to be carried out with indigo and/or derivatives thereof.

Another object of the present invention is the use according to claim 20, namely the use of an immobilized fusion enzyme in the dyeing process of textiles, wherein said immobilized fusion enzyme is a regenerating enzyme-oxidizing enzyme fusion enzyme, preferably is the PTDH-mFMO fusion enzyme, *i.e.* the immobilized fusion enzyme according to the previous object of the invention.

Said immobilized fusion enzyme, in particular said immobilized PTDH-mFMO fusion enzyme, has been revealed particularly useful in dyeing processes (such as the dyeing process of the invention) wherein insoluble dyes, in particular wherein indigo and/or derivatives thereof, are used as dyes. Indeed, good reaction rates and yields in the oxygenation of first dye precursor, in particular of indole and/or derivatives thereof, are provided by said immobilized fusion enzyme, in particular by said immobilized PTDH-mFMO fusion enzyme. Therefore, said immobilized fusion enzymes, in particular said immobilized PTDH-mFMO fusion enzymes, are optimal for synthesizing insoluble dyes, in particular indigo and/or derivatives thereof, and to carry out dyeing processes according to the invention.

A further object of the present invention is a method according to claim 21, namely a method for the production of indigo or an indigo derivative by enzymatic synthesis that comprises the following steps:

- a') converting tryptophan or a tryptophan derivative in the presence of at least a tryptophanase, to obtain indole or an indole derivative;
- b') hydroxylating said indole or said indole derivative obtained in step a') in the presence of at least an oxidizing enzyme, to obtain indoxyl or an indoxyl derivative; and
- c') converting said indoxyl or said indoxyl derivative obtained in step b') to indigo or an indigo derivative.

The reaction scheme of the method of the invention is represented in Scheme 6 above.

The method of the invention provides the synthesis of indigo or an indigo derivative starting from tryptophan or a tryptophan derivative, by means of enzymatic cascade reaction steps (steps a') and b')) and a non-enzymatic step (step c')). The method of the invention is particularly advantageous to produce indigo and/or indigo derivatives, such as Tyrian purple, in a cost effective way.

Also, advantageously, the method of the invention allows the manufacturing of indigo and/or indigo derivatives on an industrial scale.

As used herein, "tryptophan derivative" refers to a tryptophan that is substituted by the groups and the positions as defined above with reference to indole and indoxyl derivatives.

The tryptophan derivative of step a') is preferably a tryptophan halogenated derivative, which can be obtained by halogenating tryptophan in the presence of at least a tryptophan halogenase and a halogen source. The reaction scheme of this embodiment is represented in Scheme 7 above (wherein the halogenated derivatives are the 6-bromo derivatives).

According to embodiments, tryptophan, can be used as a starting compound to enzymatically produce indigo and indigo derivatives. Advantageously, the use of tryptophan as starting compound allows for a cost effective production of indigo and/or indigo derivatives.

As used herein, "halogenated derivative" refers to any tryptophan, indole, indoxyl and indigo substituted by a halogen group, in particular fluoro, chloro, bromo or iodo groups, on one or more carbons in position 5, 6, 7 and 8 (and also 5', 6', 7' and 8' for indigo). For example, tryptophan halogenated derivatives are 6-bromotryptophan (compound IVd of Scheme 7 above) and 7-chlorotryptophan, indole halogenated derivatives are 6-bromoindole (compound IId of Scheme 7 above) and 7-chloroindole, indoxyl halogenated derivatives are 6-bromoindoxyl (compound IIId

of Scheme 7 above) and 7-chloroindoxyl, and indigo halogenated derivatives are Tyrian purple (6,6'-dibromoindigo, compound Id of Scheme 7 above) and 7,7'-dichloroindigo.

According to embodiments, the enzymes employed in the method of the invention, as well as in the process of the invention, may be isolated enzymes, preferably as purified or semi-purified, whereby the method of the invention can be carried out without them being expressed in bacteria. Preferably, said isolated enzymes are immobilized enzymes.

The method of the invention can be carried out in one reactor, whereby providing a one pot reaction. This provide indigo or the derivative thereof as a solid precipitate after step c'), which can be isolated (*e.g.* filtered) from the reaction mixture. In other words, when the method of the invention is carried out according to a one pot reaction, the indigo or the derivative thereof obtained may be isolated and purified from the reaction mixture.

According to embodiments, step c') may be performed in the presence of a textile, so that at least part of the indigo or indigo derivatives that is obtained is deposited onto the textile.

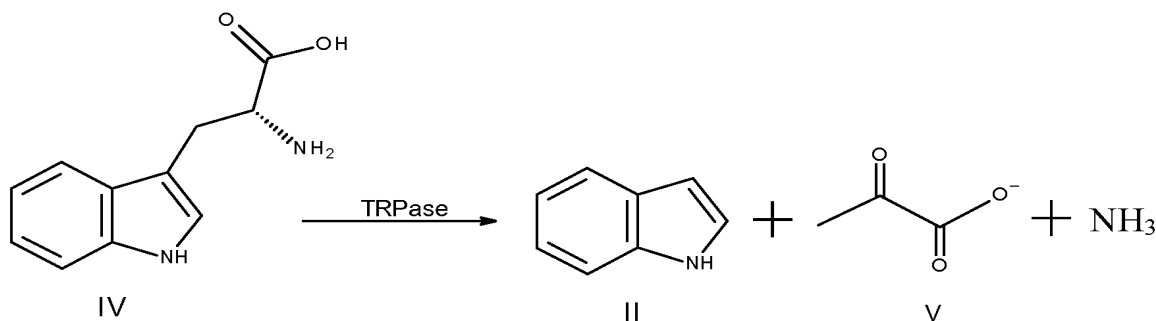
According to embodiments, steps a') to c') are carried out in an aqueous medium and a flow of the aqueous medium is generated, whereby steps a') to c') are carried out in different reactors or in different locations of a reactor. This embodiment is advantageous for carrying out each step according to its optimal parameters, such as temperature, pH, amount of enzymes' substrates, etc.

According to exemplary embodiments, the method of the invention may be carried out in a two-stage packed-bed reactor, such as Spinchem® Rotating Bed Reactor (RBR).

The method of the invention is preferably carried out in an aqueous medium. Such aqueous medium has preferably a neutral or slightly basic pH, such as 7.0 to 10, preferably 7.4 or 8. Such aqueous medium can thus comprise a buffering agent, for example a potassium phosphate buffer. Some tryptophan derivative, such as 6-bromotryptophan, are poorly soluble in aqueous medium, and the method of the invention can be carried out with such tryptophan derivatives suspended in the aqueous medium.

Step a') involves the cleavage of a carbon-carbon bond on tryptophan or on the derivative thereof in the presence of a tryptophanase. Such tryptophan derivative is preferably synthesized by carrying out step a').

The reaction step of step a') is represented in Scheme 8:



Scheme 8

wherein compound IV is tryptophan, compound II is indole, compound V is pyruvate and TRPase is tryptophanase. It has been observed that tryptophanase can be used to catalyze the conversion of tryptophan derivatives to indole derivatives, such as the conversion of 6-bromotryptophan to 6-bromoindole.

Tryptophanases (systematic name: L-tryptophan indole-lyase (deaminating; pyruvate-forming)) are known enzymes that cleave a carbon-carbon bond of tryptophan, releasing indole. They may use pyridoxal phosphate (PLP) as cofactor. A tryptophanase suitable to be used in the method of the invention the tryptophanase of *Escherichia coli* NEB® 10β.

PLP can be optionally added to the reaction mixture of step a') to improve the yield of the conversion of tryptophan or of its derivatives.

Step b') of the invention involves the hydroxylation at least on the carbon in position 3 of the indole or its derivative obtained from step a') in the presence of oxidizing enzyme and O<sub>2</sub>. Step b') thus provide indoxyl or its derivative.

Suitable oxidizing enzymes are the ones as described above, *e.g.* microbial FMO from *Methylophaga sp* strain SK1 and Baeyer-Villiger monooxygenase.

Oxidizing enzymes require O<sub>2</sub>, *i.e.* oxygen, within the reaction mixture in order to catalyze the hydroxylation of indole or its derivative. The O<sub>2</sub> required for carrying out step b') can be the oxygen normally dissolved within the aqueous reaction mixture, or the reaction mixture can be saturated with O<sub>2</sub> in order to achieve maximum conversion of indole or its derivatives.

Step c') is non-enzymatic and involves the oxidation and dimerization of indoxyl or its derivatives to indigo or its derivatives.

According to embodiments, step c') of the method of the invention may be carried out in presence of a textile, which is spaced apart at least from said oxidizing enzyme, so that the production of indigo or indigo derivatives occurs directly onto the textile, whereby at least part of said textile is dyed. In this case, advantageously, precipitation of indigo or its derivative at and/or near the enzymes is substantially avoided.

Step c') can occur spontaneously after step b') (provided that O<sub>2</sub> concentration is in an adequate amount to oxidize indoxyl or its derivatives), or it can be driven (*e.g.* by adding O<sub>2</sub> to the reaction mixture).

The O<sub>2</sub> required to carry out step c') can be the oxygen normally dissolved within the aqueous reaction mixture, or the reaction mixture can be saturated with O<sub>2</sub> in order to achieve maximum conversion of indole or its derivatives.

In an embodiment, the tryptophan derivative of step a') is a tryptophan halogenated derivative that is obtained by a further step of i) halogenating tryptophan in the presence of at least a tryptophan halogenase.

Tryptophan halogenases are known enzymes able to catalyze the halogenation of tryptophan in various positions. Tryptophan halogenases are usually flavin-dependent halogenases, *i.e.* they use FAD or FADH<sub>2</sub> as a cofactor. Suitable tryptophan halogenases according to the method of the invention are thermophilic tryptophan halogenases, such as the thermophilic tryptophan halogenase of *Streptomyces violaceusniger*.

According to embodiments, the tryptophan halogenase is the thermophilic tryptophan halogenase of the strain SPC6 of *Streptomyces violaceusniger*.

For example, the thermophilic tryptophan halogenase may have the following sequence:

LNNVVIVGGGTAGWMTASYLKAAFGDRIDITLVESGHIGAVGVGEATFSDIRHFFEFLG  
LKEKDWMPACNATYKLAVRFENWREKGHYFYHPFEQMRSVNGFPLTDWWLKQGPTD  
RFDKDCFVMASVIDAGLSPRHQDGTLDQPFDEGADEMQGLTMSEHQGKTQFPYAYQF  
EAALLAKYLTKYSVERGVKHIVDDVREVSLDDRGWITGVRTGEHGDLTGDLFIDCTGF  
RGLLLNQALEEPFISYQDTLPNDSAVALQVPMDMERRGILPCTTATAQDAGWIWTIPLT  
GRVGTGYVYAKDYLSPEEAERTLREFVGPAADVEANHIRMRIGRSRNSWVKNCVAIG  
LSSGFVEPLESTGIFFIHHAIEQLVKNFPAADWNSMHRDLVNSAVSHVMDGVREFLVLH  
YVAAKRNDTQYWRDTKTRKIPDSLAERIEKWKVQLPDSETVYPYHGLPPYSYMCILL  
GMGGIELKPSPALALADGGAAQREFEQIRNKTQRLTEVLPKAYDYFTQ (SEQ. ID NO. 1).

This type of tryptophan halogenase catalyze preferably the halogenation on the carbon in position 6 of tryptophan, whereby it is suitable to produce Tyrian purple (6,6'-dibromoindigo) according to the method of the invention.

Another tryptophan halogenase suitable for the method of the invention is tryptophan halogenase PrnA, preferably is the PrnA of *Pseudomonas fluorescens*, which catalyzes preferably the halogenation of tryptophan on the carbon in position 5 or 7 of the tryptophan.

For example, the tryptophan halogenase (PrnA) may have the following sequence:

MNKPIKNIVIVGGGTAGWMAASYLVRALQQQVNITLIESAAIPRIGVGEATIPSLQKVFF  
 DFLGIPEREWMPQVNGAFKAAIKFVNWRKPPDHSRDDYFYHLFGSVPNCDGVPLTHYW  
 LRKREQGFQQPMEYACYPQPGALDGKLAPELLDGTQMSHAWHFD AHLVADFLKRW  
 AVERGVNRVVDEVVEVRLNDRGYISTLLTKEGRTLEGDLFIDCSGMRGLLINQALKEPFI  
 DMSDYLLCDSAVASAVPNDDVREGVEPYTSAIAMNSGWTWKIPMLGRFGSGYVFSSKF  
 TSRDQATADFLNLWGLSDNQSLNQIKFRVGRNKRAWVNNCVSIGLSSCFLEPLESTGIYF  
 IYAALYQLVKHFPDTSFDPRLSDAFNAEIVYMFDDCRDFVQAHYFTTSREDTPFWLANR  
 HELRLSDAIKEKVQRYKAGLPLTTTSFDDSTYYETFDYEFKNFWLNGNYCIFA GLGML  
 PDRSLPLLQHRPESIEKAEAMFASIRREAERLRTSLPTNYDYLRSLRNGDAGQSRNQ RGP  
 TLAAKEGL (SEQ. ID. NO. 2).

According to embodiments, the tryptophan halogenase may be a genetically modified enzyme; in other words, the tryptophan halogenase may be in a mutant form. For example, the tryptophan halogenase may be a mutant form of the tryptophan halogenase of the strain SPC6 of *Streptomyces violaceusniger*, or a mutant form of the tryptophan halogenase PrnA.

The present embodiment requires a halogen source within the reaction mixture in order to be carried out, as tryptophan has to react with a halogen in presence of tryptophan halogenase to be converted to an indigo halogen derivative. Suitable halogen sources according to the method of the invention can be halogen salts, *i.e.* salts wherein the anion is halide ion. Suitable halogen salts can be magnesium, silver, sodium, potassium, lithium, and calcium halogen salts, for example NaCl, KCl, KI, LiCl, CuCl<sub>2</sub>, CuBr<sub>2</sub>, AgCl, CaCl<sub>2</sub>, CaBr<sub>2</sub>, ClF, MgCl<sub>2</sub>, MgBr<sub>2</sub>, etc.

The present embodiment is advantageously carried out at temperature comprised in the range from 20 °C to 60 °C, preferably from 25 °C to 40, more preferably at about 30 °C, for a time comprised in the range of from 30 minutes to 4 hours, preferably from 1 hour to 3 hours, more preferably for about 2 hours.



According to embodiments, cofactor regenerating enzymes may be used to regenerate the cofactor(s) which may be needed by the enzymes used in the method of the invention.

According to embodiments, step b') may be carried out in the presence of at least an enzyme suitable for regenerating NADPH cofactor. Preferably, the enzyme suitable for regenerating NADPH cofactor is selected from the group consisting of glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH) as described below, more preferably is PTDH as described below, whereby a FMO- NADPH regenerating enzyme system is provided. Advantageously, this embodiment provides for an enzyme system wherein expensive cofactors (*i.e.* NADPH) are regenerated by consuming cheaper cofactors (such as glucose, phosphite or formate). For example, oxidizing enzymes such as FMOs may use NADPH as cofactor which may be produced by the NADPH regenerating enzyme that uses cheap cofactors such as glucose, phosphite and formate.

In another embodiment, the halogenation of tryptophan to obtain its halogenated derivative is carried out in the presence of a flavin reductase and a NAD(P)H regenerating enzyme, preferably selected from the group consisting of glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH), more preferably is PTDH, whereby a tryptophan halogenase-flavin reductase-NAD(P)H regenerating enzyme system is provided.

Flavin reductases (EC 1.5.1.30) are enzyme that catalyzes the following reaction:



while NAD(P)H regenerating enzymes are enzymes that produce NADH or NADPH, such as GDH, PTDH and FDH. Advantageously, this embodiment provides for an enzyme system wherein expensive cofactors (*i.e.* FAD and NADH or NADPH) are regenerated by consuming cheaper cofactors (such as glucose, phosphite or formate), improving the industrial feasibility of the method of the invention. For example, tryptophan halogenase may use FAD as cofactor which may be produced by the flavin reductase that may use NADH or NADPH as cofactor, which is produced by the NAD(P)H regenerating enzyme that uses cheap cofactors such as glucose, phosphite and formate.

Suitable flavin reductases useful for the method of the invention can be the flavin reductases of *Bacillus subtilis*, in particular the flavin reductases of the strain WU-S2B of *Bacillus subtilis*.

For example, the flavin reductases may have the following sequence:

MKVLVLAFHPNMEQSVVNRAFADTLKDAPGITLRDLYQEYPDEAIDVEKEQKLC EEHD  
RIVFQFPLYWYSSPPLLKKWLDHVLLYGWAYGTNGTALRGKEFMVAVSAGAPEEAYQ  
AGGSNHYAISELLRPFQATSNFIGTTYLPPYVFYQAGTAGKSELAEGATQYREHVLKSF  
(SEQ. ID NO. 3).

5 Mutant forms of any enzyme employed in the method of the invention can be used to improve yields and industrial feasibility of the method of the invention.

For example, FMO mutations selected from the group consisting of W319A, C78I, C78I Y207W, and C78I Y207W W319F were found out to improve the catalyzing activity of FMO on 6-bromoindole. Moreover, the NADPH regenerating enzyme can be a mutant that has improved  
10 NADPH production, *e.g.* PTDH disclosed in WO 2004/108912 A2.

According to embodiments, when an enzyme requires a co-factor, such enzyme may be provided as a fusion enzyme with the co-factor-regenerating enzyme.

For example, tryptophan halogenase and flavin reductase can be provided as a fusion enzyme, and FMO and NADPH regenerating enzyme can be provided as a fusion enzyme, preferably as PTDH-  
15 FMO. According to this embodiment, only three individual enzymes can be employed in the method of the invention (when optional step i) is carried out), namely a tryptophan halogenase-flavin reductase fusion enzyme, a tryptophanase and a FMO-NADPH regenerating fusion enzyme. The NADPH regenerating portion of the latter fusion enzyme is able to regenerate the NADPH required for both the FMO region and the flavin reductase region of the fusion enzyme starting  
20 from its cheap substrate, *i.e.* phosphite.

Still a further object of the present invention is a dyed textile as obtainable through the process of the invention.

Also object of the present invention is a dyed textile as obtainable through the method of the invention when step c') of the method is carried out in the presence of a textile.

## 25 DESCRIPTION OF THE FIGURES

Figure 1 is a schematic view of the process of the invention.

Figure 2 schematically shows another embodiment of the process of the invention;.

Figure 3 schematically shows an embodiment of the process;

Figure 4 is a schematic view of the apparatus 10.

30 Figure 5 is another schematic view of the apparatus 10.

Figure 6 schematically shows an embodiment of the apparatus 10, including a reservoir;

Figure 7 schematically shows a further embodiment of the apparatus 10, including a collection tank;

Figure 8 schematically shows an embodiment of the apparatus 10; and

5 Figure 9 schematically shows another embodiment of the apparatus 10.

## DESCRIPTION OF THE INVENTION

The objects and embodiments of the invention are now disclosed in more details with reference to the figures.

10 Object of the present invention is a process for dyeing a textile 22, comprising an enzymatic synthesis of a dye precursor, characterized in that it comprises the following steps:

- a) contacting a solution comprising at least a first dye precursor 112 with at least a first immobilized enzyme 12, to convert at least part of said at least a first dye precursor 112 into at least a second dye precursor 113, to obtain a solution comprising said at least second dye precursor 113;
- 15 b) generating a flow of said solution comprising said at least second dye precursor 113, whereby said solution comprising said at least second dye precursor 113 flows from said first immobilized enzyme 12 to said textile;
- c) contacting said solution comprising said at least second dye precursor 113 with said textile 22; and
- 20 d) converting at least part of said second dye precursor 113 to at least a dye 111, whereby at least part of said textile 22 is dyed;

wherein said at least first immobilized enzyme 12 is spaced apart from said textile 22, for example as represented on Figure 1.

25 Figure 1 represents a schematic view of the process of the invention, in particular is represented the conversion of said first dye precursor 112 to said second dye precursor 113 by means of said first immobilized enzymes 12, then the flow of the solution comprising at least said second dye precursor 113 to the textile 22 (which is spaced apart from said first immobilized enzyme 12), and finally the conversion of said second dye precursor 113 to said insoluble dye 111 directly onto said textiles 22.

30 More in particular, with reference to Figure 1, the process of the invention will be disclosed with reference to the indole to indigo route. The process provides the dyeing of textiles 22 as a result of

the following steps: a flow of a solution comprising indole dye precursor 112 is contacted with at least a first immobilized enzyme 12 or an enzymatic system (step a)), thus obtaining the conversion of at least part of indole into indoxyl 113, due to enzymatic catalysis. The solution is now comprising indoxyl 113. The flow of solution allows contact of solution comprising indoxyl 113 with the textile 22; advantageously, parameters, such as flow rate, are controlled in order to have the solution reaching said textile 22 immediately or shortly after indoxyl 113 is synthesized. Conversion of indoxyl 113 to indigo 111 can be thus obtained onto said textile 22, and dyeing of said textile 22 is achieved. In particular, when said solution comprising indoxyl 113 wets said textile 22, at least part of said indoxyl 113 is converted into indigo 111 directly onto said textile 22.

According to the present invention, the process provides for generating a flow of the solution initially to the enzymes 12 (or enzyme system) and then from the enzymes 12 (or enzyme system) to the textile 22, for example as shown by Figure 1, wherein the straight arrows represent the direction of the flow of solution. Any direction of the flow of solution is possible, provided that such flow of solution is first fed to said enzymes 12, and then from said enzymes 12 to said textile 22. For example, another direction of the flow of solution can be from the bottom to the top when the first immobilized enzymes 12 are below said textile 22, or can be from top to bottom when the first immobilized enzymes 12 are above said textile 22, or can be circular when the first immobilized enzymes 12 and said textile 22 are housed within different areas of an annular chamber, such as a toroidal chamber, for example, when they are held in diametrically opposed areas within such toroidal chamber.

The use of a flow in the process of the invention prevents the precipitation of indigo 111 at and/or near the immobilized enzymes 12, as the solution comprising at least said second dye precursor 113 flows to said textile 22, which is spaced apart from said first immobilized enzyme 12, and then conversion of said second dye precursor 113 into said insoluble dye 111 occurs.

In particular, when said second dye precursor 113 can spontaneously convert into say insoluble dye 111, for example when said second dye precursor 113 is indoxyl and/or derivatives thereof and said insoluble dye 111 is indigo and/or derivatives thereof, a flow of the solution comprising at least said second dye precursor 113 is generated whereby said solution flows to said textile 22 before said second dye precursor 113 spontaneously converts and precipitate as said insoluble dye 111 at and/or near the immobilized oxidizing enzyme.

When said second dye precursor 113 does not spontaneously convert into said insoluble dye 111, or when it does not spontaneously convert in suitable amount, it is possible to change conditions, such as pH and/or temperature, and/or to add reagents, and/or supply with gasses, such as oxygen, where the textile 22 and said second dye precursor 113 are, in order to drive the conversion of said second dye precursor 113 to said insoluble dye 111 on the textile 22. Vice versa, the solution conditions may be controlled also in step a) of the process to prevent conversion until the solution has reached the textile.

Moreover, two or more different first dye precursors 112 can be contacted with the enzymes 12 according to step a) of the process, thus obtaining two or more different second dye precursors 113. Such two or more different second dye precursors 113 can then convert according to step d) of the process to obtain one or more different dyes 111 onto the textile 22; eventually, the conversion of step d) of such two or more different second dye precursors 113 can be achieved by adding further reactants, and/or by changing the parameters of the solution comprising them, if such conversion does not occur spontaneously, or if it does not occur in suitable amount. An illustrative example wherein two or more different dye precursors are contacted with the enzymes 12 is represented in Scheme 5 above. When two or more different first dye precursors 112 are used according to the process of the invention, two or more different enzymes 12 may be required; for example, when the two or more different first dye precursors 112 require different enzymatic reaction to be converted to the respective two or more different second dye precursors 113, or when the two or more different first dye precursors 112 cannot be substrates of the same enzymes 12.

The solution comprising at least a first dye precursor 112 can comprise also other solutes and is the solution that is contacted with the first immobilized enzyme 12 or the enzyme system in step a) of the process. The solution comprising at least a second dye precursor 113 is the solution obtained after at least part of said first dye precursor 112 is converted into said second dye precursor 113 after step a) and may comprise also other solutes, *e.g.* some unreacted first dye precursor 112.

As above mentioned, the solution according to the invention may comprise other functional solutes, such as salts, buffering agents, co-factors and oxygen and/or peroxide scavengers (*e.g.* catalases). Preferably, the concentration of substrates comprised in the aqueous solution saturate said catalyzing enzymes, so that said first immobilized enzymes can effectively catalyze a conversion of said first dye precursor 112 to the second dye precursor 113. An illustrative solution according to the present invention may comprise 100 mM potassium phosphate buffer pH 8.0, 0.5

M NaCl, 100 microM NADPH, 20 mM sodium-phospite and 1 nanoM bovine liver catalase with water as a solvent, when said first dye precursor 112 is indole, said first immobilized enzyme 12 is mFMO, and said second immobilized enzyme is PTDH.

5 The process of the invention can provide dyeing of textile 22 in batch or in continuous. To carry out the latter, addition of said first dye precursor 112, for example to the solution before step a), is required, so that a solution comprising at least a first dye precursor 112 is continuously contacted with said first immobilized enzymes 12 and the second dye precursor 113 is continuously synthesized. Said first dye precursor 112 is advantageously added keeping said first immobilized enzymes 12 saturated. Also addition of other solutes, for example before step a), could be required  
10 to carry out the process of the invention in continuous, such as cofactor(s), buffering agents, and oxygen.

Temperature and pH values according to the process of the invention can vary and can be those conventionally used in enzymatic synthesis of insoluble dyes.

15 The temperature of the solutions according to the process of the invention can be, for example, in the range comprised from 20 to 40, preferably from 25 to 30. pH of the solutions according to the process of the invention can be, for example, in the range comprised from 7.0 to 10.0, preferably from 7.5 to 9.0, even more preferably from 7.5 to 8.5, and most preferably is 8.0.

The contact time between said first immobilized enzyme 12 and said solution comprising indole 112 in step a) can be varied to achieve different shading of the dyed textile, and can be varied *e.g.*  
20 by varying the flow rate of the solution.

Oxygen concentration in the solution can be a relevant parameter for the overall dyeing process yield, as oxygen can take part in the conversion of the first dye precursor into the second dye precursor, and/or in the conversion of the second dye precursor into the insoluble dye (for example when the first dye precursor is indole and/or derivatives thereof, the second dye precursor is  
25 indoxyl and/or derivatives thereof, and the insoluble dye is indigo and/or derivatives thereof). Oxygen concentration in the solution can thus vary, for example, based on the amount of insoluble dye to be synthesized, or by the amount of textile to be dyed. The solution is advantageously saturated with oxygen in order to achieve maximum conversion of, for example, indole and/or derivatives thereof, and of indoxyl and/or derivatives thereof. Oxygen concentration is also  
30 advantageously monitored and controlled, and oxygen can be added when required in order to keep the solution saturated.

Other parameters of the process of the invention can be chosen according to, for example, which type of textile has to be dyed and which dye is chosen as a final dye.

According to an embodiment of the process of the invention, a flow of a solution, a so called “exhaust solution”, resulting from step d) is directed back to the chamber or areas housing the immobilized enzyme system. The exhaust solution is the solution obtained after at least part of indoxyl 113 is converted into indigo 111, which is fixed on said textiles 22, and may comprise unreacted first dye precursor 112, *e.g.* unreacted indole, in particular if said first dye precursor 112 did not react completely with said enzyme 12 in step a) of the process invention. This embodiment is shown in Figure 2. The exhaust solution comprising indole is fed back directly or indirectly, continuously or batch-wise, to the enzyme system to react the remaining indole.

Preferably, the process provides a step of adding some first dye precursor 112 to the exhaust solution. Said first dye precursor 112 is advantageously added keeping said first immobilized enzymes 12 saturated. This can provide the process of the invention carried out in continuous.

In an embodiment of the invention, said first immobilized enzyme 12 is an oxidizing enzyme as previously defined. Use of oxygenases is particularly useful when said first dye precursor 112 has to be oxidized in order to be converted to said second dye precursor 113, *e.g.* when said first dye precursor 112 is indole and/or derivatives thereof, said second dye precursor 113 is indoxyl and/or derivatives thereof, and said insoluble dye 111 is indigo and/or derivatives thereof.

The process of the invention may further provide for a second immobilized enzyme, preferably an immobilized cofactor-regenerating enzyme as previously defined, to be present in the plant for dyeing. This provide an enzyme system wherein the first immobilized enzyme catalyzes the conversion of the first dye precursor 111 and the immobilized cofactor-regenerating enzyme regenerates the co-factor(s) needed by the first dye precursor 111. The carriers used to immobilize said second immobilized enzymes may be the same as the ones used to immobilize said first immobilized enzymes or may be different, depending on the surface-exposed groups of said second enzymes. If possible, the same carriers are used to immobilize both said first and second enzymes.

The type of said immobilized cofactor-regenerating enzyme depends on which cofactor(s) is used by said first immobilized enzyme 12. For example, when the first immobilized enzymes 12 are flavin-containing monooxygenases (FMO) (which use NADPH as cofactor), the immobilized cofactor-regenerating enzymes may be at least a dehydrogenase that generates NADPH, such as dehydrogenases selected from the group consisting of: glucose dehydrogenase (GDH), phosphite

dehydrogenase (PTDH), and formate dehydrogenase (FDH). PTDH are soluble NADPH-regenerating enzymes obtainable for example from *Pseudomonas stutzeri*, that use phosphite as a substrate to catalyze the production of NADPH. The use of the FMO (preferably mFMO) along with the PTDH revealed to be effective in the synthesis of many second dye precursor 113, in particular of indoxyl and/or derivatives thereof, as it provides good oxidation rates and an efficient regeneration of NADPH.

The solutions used in the process and apparatus of the invention may also comprise cofactor(s) and/or substrates of said second immobilized enzymes. For example, if GDH, or PTDH, or FDH, is used as an immobilized cofactor-regenerating enzyme, the solutions may comprise also, respectively glucose, or phosphite salts, or formate (*i.e.* the substrates of, respectively, GDH, PTDH, and FDH).

Similarly to the first enzymes, mutant second enzymes (*e.g.* genetically modified second enzyme) can be employed in the process of the invention, for example to improve the regeneration of the desired cofactor(s) or to improve their binding properties to the carrier(s).

In an embodiment of the present invention, said first enzyme 12 and said second enzyme are provided as a fusion enzyme as previously defined. This provides an enzyme system.

With reference to Figure 3, another embodiment of the process of the invention is illustrated. Such embodiment provides for the production of the first dye precursor 112, *e.g.* indole, starting from one starting compound 114, *e.g.* tryptophan. A further step is comprised in the process of the invention according to the present embodiment, namely: contacting a solution comprising at least a starting compound 114 with at least a starting enzyme 14 to convert at least part of such starting compound 114 into the first dye precursor 112 to obtain a solution comprising such first dye precursor 112 so that steps a) to d) of the process of the invention can be carried out as already disclosed with reference to Figures 1 and 2. When the first immobilized enzyme 12 and the starting enzyme 14 are spaced apart, in order to contact the solution comprising the first dye precursor 112 (obtained by converting the starting compound 114) with the enzyme 12, a flow of such solution may be generated whereby such solution flows from the starting enzyme 14 to the first immobilized enzyme 12, for example as represented in Figure 3. As the exhaust solution resulting from step d) may also comprise unreacted starting compound 114, circulating the exhaust solution back to the reactors or areas housing the starting enzymes 14 and/or the first chamber or areas housing the first immobilized enzymes 11 may be advantageous to optimize the conversion of unreacted starting compound 114 and/or first dye precursor 112.



In a further embodiment of the process of the invention, step a) is carried in a first chamber 11, and the dyeing of textile 22 is carried out in a second chamber 21. A solution comprising the first dye precursor 112 is provided inside the first chamber 11 whereby said solution contacts the first enzyme 12 contained in said first chamber 11 and the first dye precursor 112 is converted enzymatically to the second dye precursor 113. A flow of the solution is then generated and the solution, now comprising at least said second dye precursor 113, flows into said second chamber 21 containing textiles 22, whereby the solution and the second dye precursor 113 can contact and impregnate said textile 22. Finally, the second dye precursor 113 converts directly onto the textile 22 into the dye 111.

The flow of the solution is generated by any means suitable, *e.g.* a pump 50 or gravity (if the first chamber 11 is located above the second chamber 21).

First chamber 11 is a container suitable to contain enzymes 12 or enzyme system including the second immobilized enzymes and aqueous solutions. First chamber 11 can comprise also means to contain and restrain said first immobilized catalyzing enzymes 12 (and eventually, the enzyme system) inside it, for example one or more filters. Said first chamber 11 may advantageously comprise one or more means or sensors to allow monitoring parameters of the solution such as pH, temperature of the solution, oxygen concentration, flow rate, etc. The dimension, shape and material of said first chamber 11 can be chosen arbitrarily according to many factors, such as the amount of textiles 22 to be dyed and the dimension and shape of said second chamber 21.

Second chamber 21 is a container suitable to contain at least said textiles 22 and aqueous solutions, and wherein conversion of said second dye precursor 113 to said insoluble dye 111 can occur. The second chamber 21 may also comprise means that holds said textiles 22 in a predetermined position to allow a faster or more complete dyeing process. Said second chamber 21 may advantageously comprise one or more means or sensors that allow monitoring parameters of the solution such as pH, temperature of the solution, oxygen concentration, flow rate, etc. The dimension, shape and material of said second chamber 21 can be chosen according to many factors, such as the amount of textiles to be dyed, and the dimension and shape of said first chamber 11.

The first chamber 11 and the second chamber 21 may be thermally regulated individually, for example by means of jackets, *e.g.* water jackets.

In Figure 4, the first chamber 11 is in fluid connection with the second chamber 21. Said fluid connection can be carried out by fluid connectors 11a such as tubes or pipes, which dimension, shape and material can be chosen by the skilled person to ensure the fluid connection between first

and second chamber, and that they are able to effectively contain, and advantageously are inert to, the aqueous solutions that circulate in said first chamber 11 and in said second chamber 21. Such dimension, shape and material may vary arbitrarily according to, for example, the amount of textile to be dyed and the flow rate required for the solution. Said fluid connectors (*e.g.* tubes or pipes) may advantageously comprise probes to measure parameters such as flow rate, temperature, solution pH, and oxygen concentration, as well as portholes, round windows, and/or doors to monitor the process of the invention and to *e.g.* take samples of the solution.

Another object of the present invention is an apparatus 10 for dyeing a textile 22 comprising a first chamber 11 containing at least a first immobilized enzyme 12 and a solution comprising at least a dye precursor, at least a second chamber 21 containing a textile 22, and means to generate a flow of a solution 50, wherein said first chamber 11 is in fluid connection with said second chamber 21 whereby said solution comprising at least a dye precursor can flow from said first chamber 11 to said second chamber 21 where at least part of said dye precursor is converted to a dye 111 to dye at least part of said textile 22, for example as represented on Figure 4. Said second chamber 21 optionally comprises also outlet means to remove said solution from said second chamber 21.

In particular, Figure 4 shows said first chamber 11 containing said first immobilized enzymes 12 and said second chamber 21 containing said textiles 22. The straight arrow shows the flow of a solution comprising at least a dye precursor from said first chamber 11 to said second chamber 21. Within said second chamber, at least part of said second dye precursor 113 is converted to an insoluble dye 111 to dye at least part of a textile 22 contained in said second chamber 21.

Figure 5 shows a schematic view of an embodiment of the apparatus 10, where the second chamber 21 has outlet means 21a, and wherein the outlet means 21a are in fluid connection with the first chamber 11. This allows the exhaust solution to be removed from said second chamber 21, and be subsequently fed to said first chamber 11 (as represented by the straight arrows of Figure 5). Outlet means 21a may be any tubes or pipes, for example as described above.

To carry out the process of the invention in continuous when said apparatus 10 is used, is useful to keep providing cofactors and substrates to the first chamber 11 and the first immobilized enzymes 12, so that they can keep producing the second dye precursor 113. For this reason, compounds (*e.g.* cofactors and substrates) are added to said first chamber 11 and/or to the exhaust solution that flows back into the first chamber 11. The apparatus 10 of Figure 5 is accordingly provided with means to carry out such addition, and comprises feeding means 11b connected to the first chamber 11 and/or feeding means 21b connected to the outlet means 21a.

In an embodiment of the apparatus 10 of the invention, a mean to generate a flow of a solution 50, such as a pump, is further comprised, allowing solutions comprised in the apparatus 10 of the invention to flow.

When the first immobilized enzyme 12 is not specific to just one substrate and thus is able to convert different first dye precursors 112, it is possible to provide different colors to the textiles 12 to be dyed just by changing the reagents fed to the apparatus 10. By changing first dye precursors 112 without changing the apparatus 10 and/or the enzymes 12 contained therein, different dyes 111 can be obtained; these dyes 111 are suitable to dye a textile 22 in the second chamber 21. For example, when the enzyme 12 contained in the first chamber 11 is the fusion enzyme PTDH-mFMO (which is able to convert indole as well as its derivatives), feeding a solution comprising indole to the apparatus 10 provides blue textiles. If in the same dyeing process a solution comprising 5-hydroxyindole replaces the solution comprising indole and is fed to the apparatus 10, a brown dye and a brown dyed textile is obtained.

Figure 6 shows another embodiment of the apparatus 10 further comprising one reservoir 31 in fluidic connection with at least said first chamber 11, so that a solution comprising a dye precursor can flow from said reservoir 31 to said first chamber 11. Figure 7 shows another embodiment of the apparatus 10 further comprising one collection tank 41 in fluidic connection with said outlet means 21a of said second chamber 21.

Reservoir 31 is any container that is able to contain aqueous solutions, such as a solution comprising at least said first dye precursor 112, such as indole and/or derivatives thereof. Reservoir 31 may be the container from which a solution comprising at least said first dye precursor 112 is fed the apparatus 10 in order to carry out the dyeing process of the invention. Therefore, reservoir 31 is advantageously set so that an operator can easily feed solutions and/or solutes therein, and its shape and dimension can be chosen accordingly. The apparatus 10 of Figure 6 also comprises means 50 to generate a flow of a solution, such as a pump, in order to allow the solution to flow from reservoir 31 to first chamber 11, as represented by the straight arrows on Figure 6, thus feeding a solution comprising first dye precursor 112 to first chamber 11.

Figure 8 shows an apparatus 10 comprising both a reservoir 31 and a collection tank 41. The apparatus of Figure 8 can be useful to carry out the dyeing process of the invention. A solution comprising at least a first dye precursor is provided in the reservoir 31. Then a flow of such solution is generated by the pump 50 and the solution flows to the first chamber 11 from said reservoir 31. Contacting of such solution with enzymes 12 is obtained within said first chamber 11. The solution, now comprising second dye precursor 113, flows from first chamber 11 to second chamber 21

wherein textiles 22 are located. At least part of said second dye precursor 113 is converted to the dye 111 to dye at least part of said textile 22 within said second chamber 21, and an exhaust solution is obtained. The exhaust solution is removed from the second chamber 21 through outlet means 21a and is collected in the collection tank 41. Collecting the exhaust solution could be advantageous, for example when such exhaust solution has to be treated to remove precipitates (such as insoluble dye 111) that might be present; such treatment can be carried out in the collection tank 41. The exhaust solution flows back to said first immobilized enzymes 12 comprised in said first chamber 11 (flowing through the reservoir 31) so that any unreacted first compound 112 in the solution can be contacted with said first immobilized enzymes 12. Solutes, such as the first dye precursor 111 and cofactors, may also be added to the solution by means of the reservoir 31. The apparatus 10 set as in Figure 8 may be also provided by other means to carry out addition of solutes, for example feeding means 11b connected to the first chamber 11 and/or feeding means 21b connected to the outlet means 21a (not shown in Figure 8).

More than one reservoirs 31 and/or more than one collection tanks 41 may be present in the apparatus 10 of the invention, and they can be set in series and/or in parallel.

In another embodiment, a reactor 51 for the production of the first dye precursor 112, *e.g.* indole, starting from one or more starting compound 114, *e.g.* tryptophan, is further comprised in the apparatus 10 of the invention, for example as represented on Figure 9. Such reactor 51 contains at least the starting enzyme 14. A solution comprising a starting compound 114 can be fed to the reactor 51 by means of feeding means 51b whereby such solution is contacted with starting enzymes 14 and the starting compound 114 is converted to the first dye precursor 112. The solution comprising the first dye precursor 112 can be then flowed to the first chamber 11 by generating a flow of such solution by means of a second pump 50b. Once the solution comprising the first dye precursor 112 is flowed to the first chamber 11, steps a) to d) of the process of the invention can be carried out. Figure 9 represents an embodiment wherein the reactor 51 is set in parallel with respect to the flow of the solution flowing from the first chamber 11 to the second chamber 21; however embodiments wherein one or more reactors 51 are set in series with respect to such flow of the solution, preferably are set upstream of the first chamber 11, are encompassed by the present invention.

## CLAIMS

1. A process for dyeing a textile (22), comprising an enzymatic synthesis of a dye precursor, characterized in that it comprises the following steps:
  - a) contacting a solution comprising at least a first dye precursor (112) with at least a first  
5 immobilized enzyme (12), to convert at least part of said first dye precursor (112) into at least a second dye precursor (113), to obtain a solution comprising said at least second dye precursor (113);
  - b) generating a flow of said solution comprising said second dye precursor (113), whereby  
10 said solution comprising said second dye precursor (113) flows from said first immobilized enzyme (12) to said textile (22);
  - c) contacting said solution comprising said second dye precursor (113) with said textile (22); and
  - d) converting at least part of said second dye precursor (113) to at least one dye (111),  
15 whereby at least part of said textile (22) is dyed;wherein said first immobilized enzyme (12) is spaced apart from said textile (22).
2. A process according to the previous claim, wherein an exhaust solution obtained in step d), and a flow of said exhaust solution is generated whereby said exhaust solution flows to said at least first immobilized enzyme (12).
3. A process according to claim 1 or 2, wherein said at least a first dye precursor (112) is indole  
20 and/or derivatives thereof, said at least a second dye precursor (113) is indoxyl and/or derivatives thereof, and said at least a dye (111) is indigo and/or derivatives thereof.
4. A process according to any previous claim, wherein said first immobilized enzyme is an oxidizing enzyme, preferably an oxygenase, more preferably a monooxygenase, whereby said first dye precursor is oxidized to obtain said second dye precursor when contacted by said  
25 oxidizing enzyme.
5. A process according to any previous claim, further comprising a second immobilized enzyme, preferably an immobilized cofactor-regenerating enzyme.
6. A process according to claim 5, wherein said cofactor-regenerating enzyme is a  
30 dehydrogenase; preferably is at least one dehydrogenase selected from the group consisting of: glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH); more preferably is a phosphite dehydrogenase (PTDH).

7. A process according to claim 5 or 6, wherein said first immobilized enzyme and said second immobilized enzyme are provided as an immobilized fusion enzyme, preferably said fusion enzyme is PTDH-mFMO.
8. A process according to any claim 4 to 7, wherein oxygen is added to said solution.
- 5 9. A process according to any previous claim, wherein step a) is carried out in at least a first chamber (11), and wherein the dyeing of at least part of said textile (22) is carried out in at least a second chamber (21).
- 10 10. A process according to claim 9, wherein said first dye precursor (112) is produced enzymatically starting from one or more starting compounds (114) in one or more reactors (51) different from said first chamber (11) and said second chamber (21).
11. A process according to claim 9, wherein said first dye precursor (112) is produced enzymatically starting from one or more starting compounds (114) in said first chamber (11).
12. An apparatus (10) for dyeing a textile (22) comprising a first chamber (11) containing at least a first immobilized enzyme (12) and a solution comprising at least a dye precursor, at least a  
15 second chamber (21) containing a textile (22), and means to generate a flow of a solution (50), wherein said first chamber (11) is in fluid connection with said second chamber (21) whereby said solution comprising at least a dye precursor can flow from said first chamber (11) to said second chamber (21) where at least part of said dye precursor is converted to a dye (111) to dye at least part of said textile (22), and wherein said second chamber (21) optionally  
20 comprises outlet means (21a) to remove said solution from said second chamber (21).
13. The apparatus (10) according to claim 12, wherein said first immobilized enzyme (12) is an oxidizing enzyme, preferably is an oxygenase, more preferably a monooxygenase, most preferably is mFMO.
14. The apparatus (10) according to claim 12 or 13, wherein said first chamber (11) further  
25 contains at least a second enzyme, preferably at least a cofactor-regenerating enzyme, more preferably is at least one dehydrogenase, even more preferably is a dehydrogenase selected from the group consisting of: glucose dehydrogenase (GDH), phosphite dehydrogenase (PTDH), and formate dehydrogenase (FDH); and most preferably is a phosphite dehydrogenase (PTDH).
- 30 15. The apparatus (10) according to claim 14, wherein said first immobilized enzyme (12) and said second enzyme are provided as an immobilized fusion enzyme.
16. The apparatus (10) according to claim 15, wherein said immobilized fusion enzyme is PTDH-mFMO.

17. The apparatus (10) according to any one of claims from 12 to 16, further comprising:

- one or more reservoirs (31) in fluid connection with at least said first chamber (11), so that a solution comprising a dye precursor can flow from said reservoir (31) to said first chamber (11); and/or
- one or more collection tanks (41) in fluid connection with said outlet means of said second chamber (21).

18. The apparatus (10) according to any one of claims from 12 to 17, wherein said outlet means (21a) of said second chamber (21) are in fluid connection with said first chamber (11).

19. An immobilized fusion enzyme comprising a carrier and at least a fusion enzyme immobilized on said carrier, wherein said fusion enzyme is the PTDH-mFMO fusion enzyme.

20. The use of the immobilized fusion enzyme according to claim 19 in the dyeing process of textile.

21. A method for the production of indigo or an indigo derivative by enzymatic synthesis that comprises the following steps:

- a') converting tryptophan or a tryptophan derivative in the presence of at least a tryptophanase, to obtain indole or an indole derivative;
- b') hydroxylating said indole or said indole derivative obtained in step a') in the presence of at least an oxidizing enzyme, to obtain indoxyl or an indoxyl derivative; and
- c') converting said indoxyl or said indoxyl derivative obtained in step b') to indigo or an indigo derivative.

22. The method according to the previous claim, wherein said tryptophan derivative of step a') is a tryptophan halogenated derivative, comprising the further step of: i) halogenating tryptophan to obtain said tryptophan halogenated derivative in the presence of at least a tryptophan halogenase and a halogen source.

23. The method according to claim 21 or 22, wherein said tryptophanase, said oxidizing enzyme, and said tryptophan halogenase are isolated enzymes, preferably immobilized enzymes.

24. The method according to any claim 21 to 23, wherein steps a') to c') are carried out in an aqueous medium and a flow of the aqueous medium is generated, whereby said steps a') to c') are carried out in different reactors or in different locations of a reactor.

25. The method according to any claim from 21 to 24, wherein said halogen bromine, said tryptophan derivative is 6-bromotryptophan said indigo derivative is Tyrian purple.

26. The method according to any claims 21 to 25, wherein step c') is carried out in the presence of a textile that is spaced apart at least from said oxidizing enzyme, whereby at least part of said textile is dyed.
27. A dyed textile as obtainable according to any claim 1 to 11 or 26.



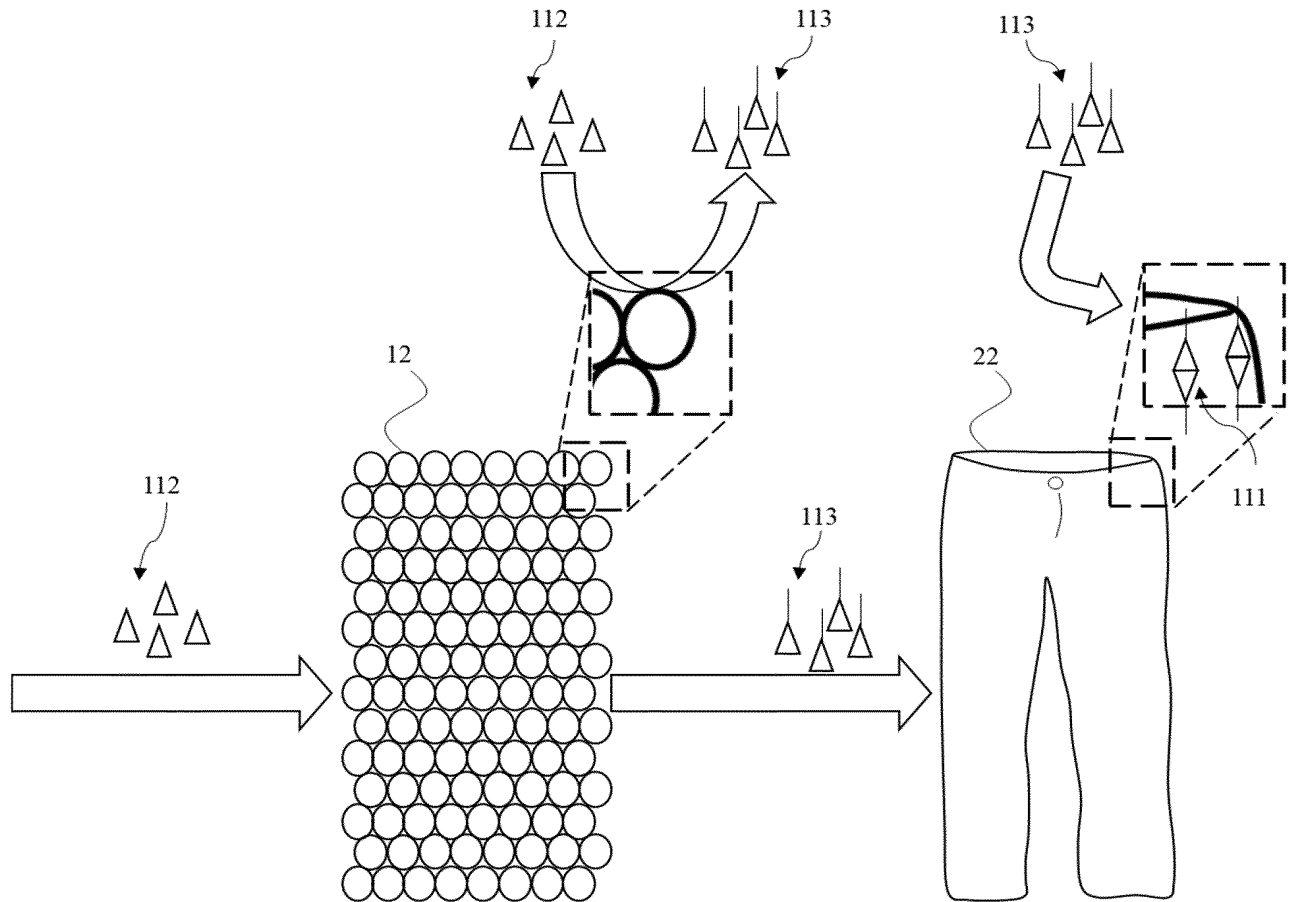


Figure 1

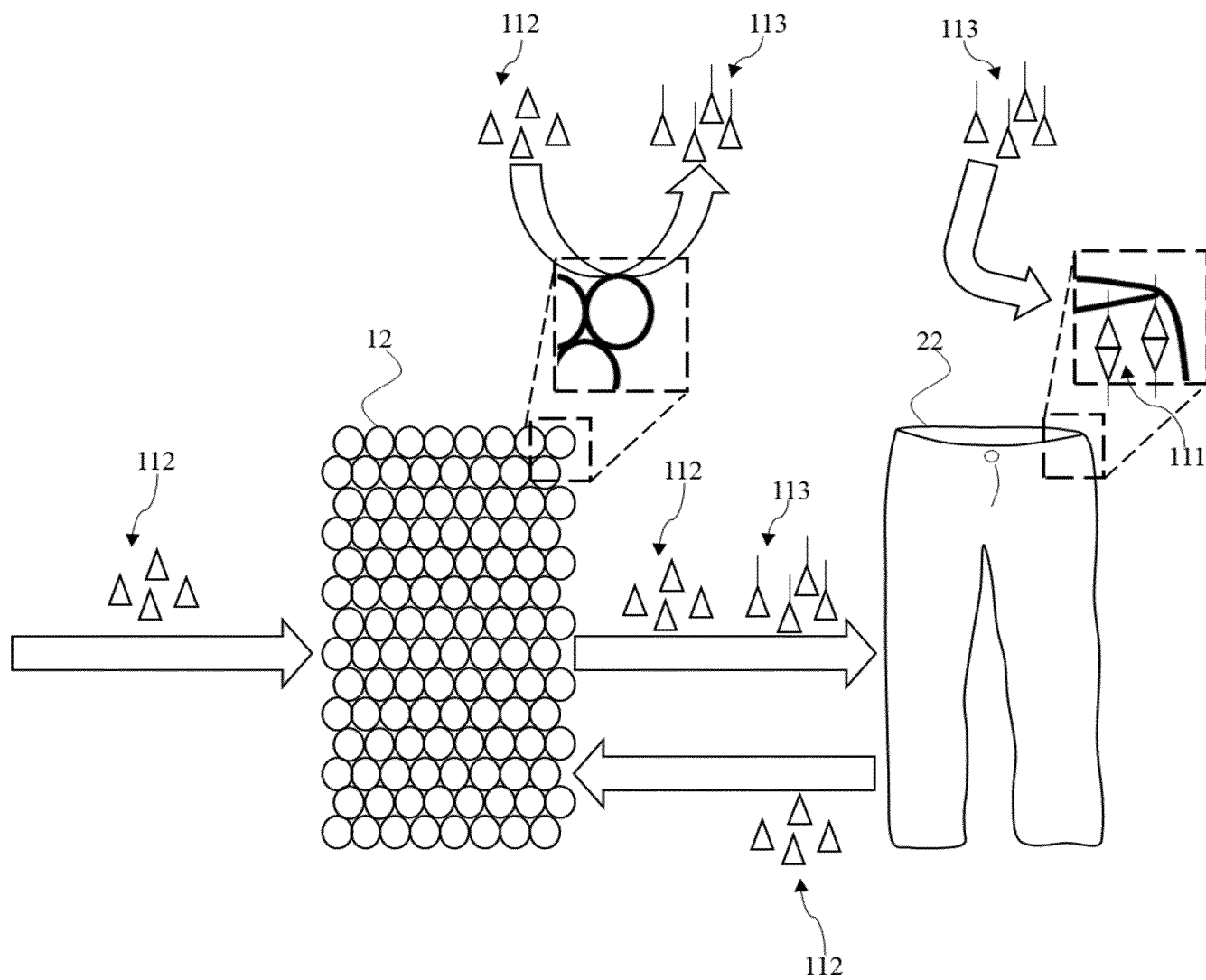


Figure 2

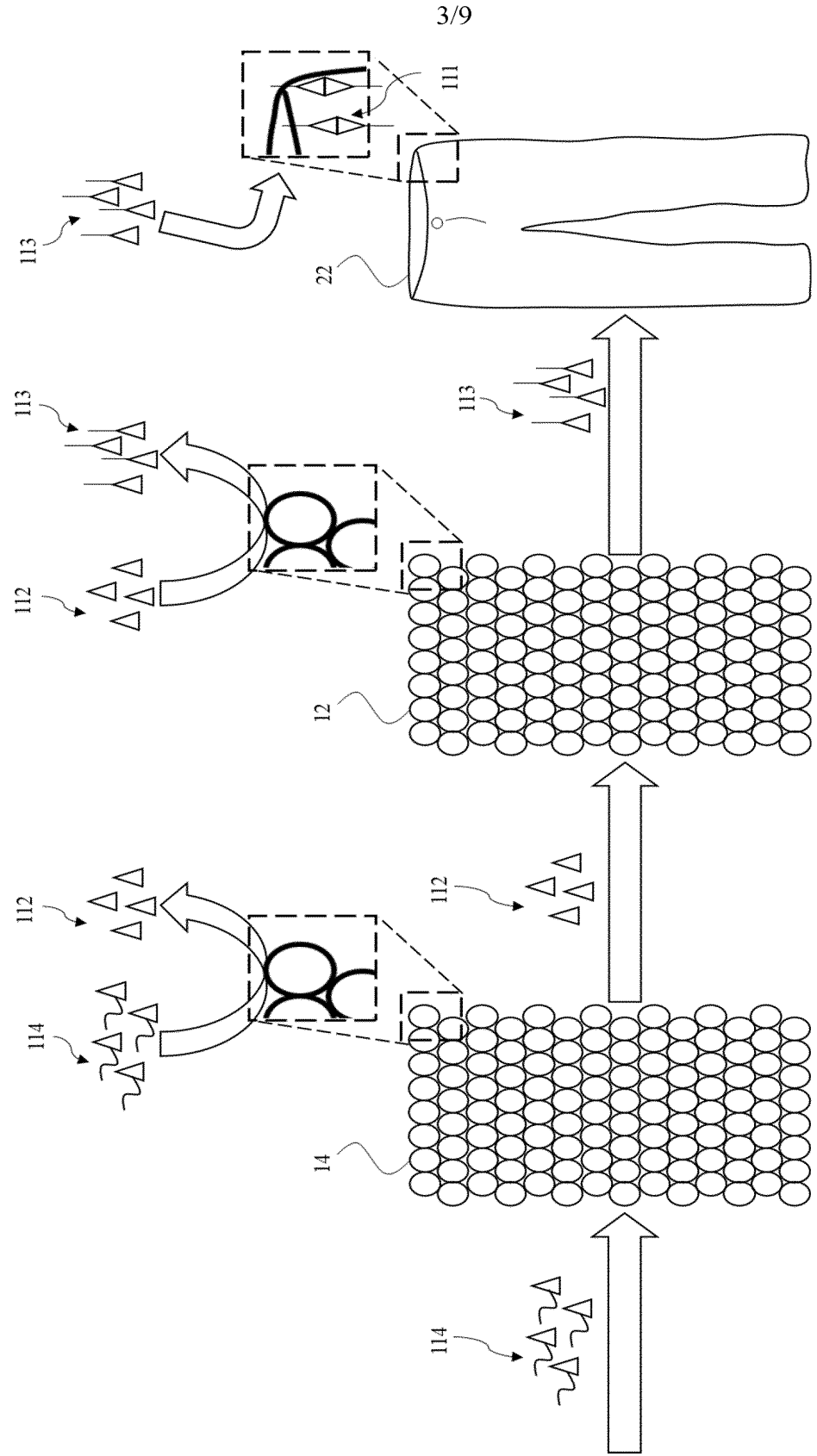


Figure 3

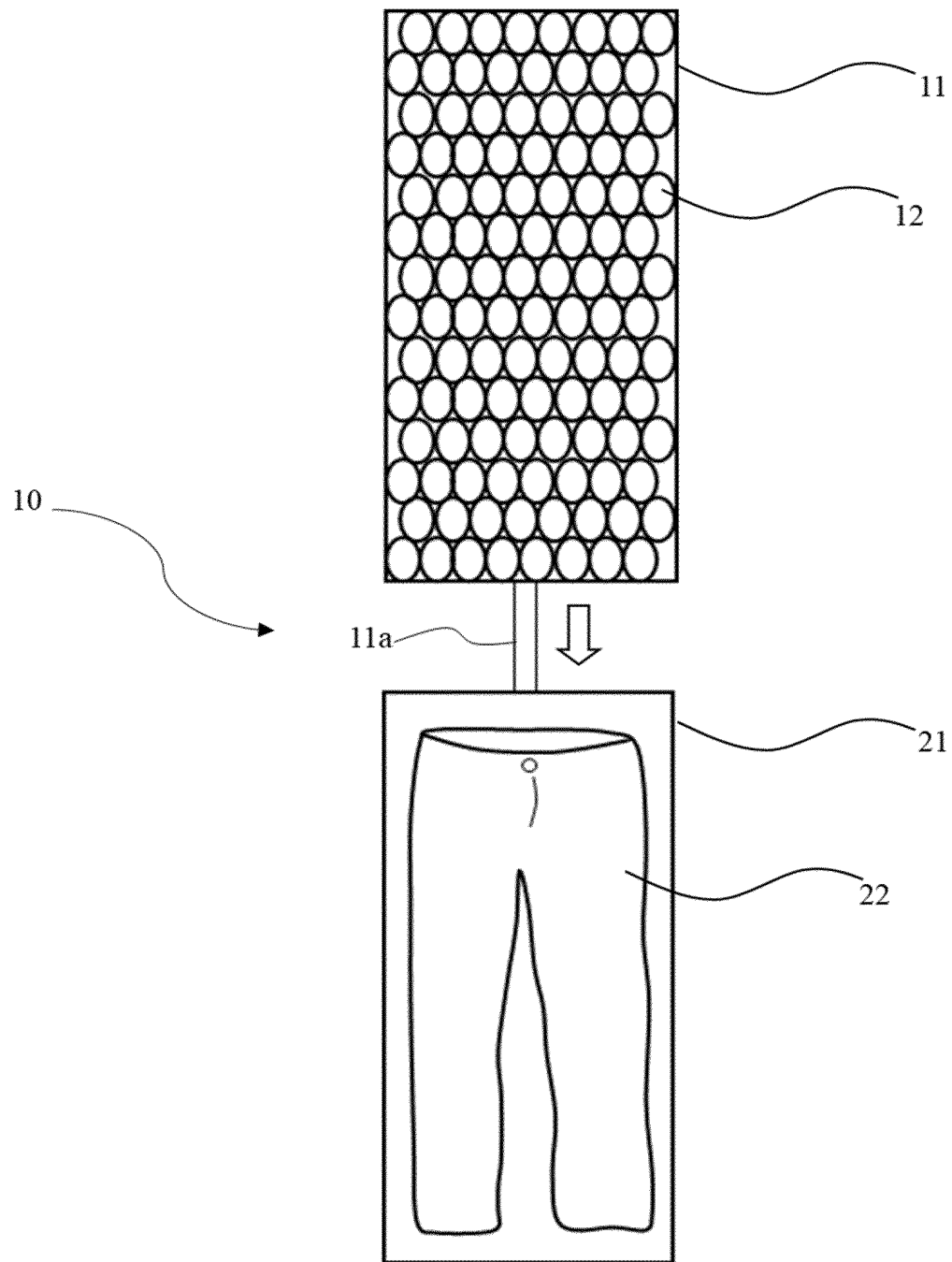


Figure 4

5/9

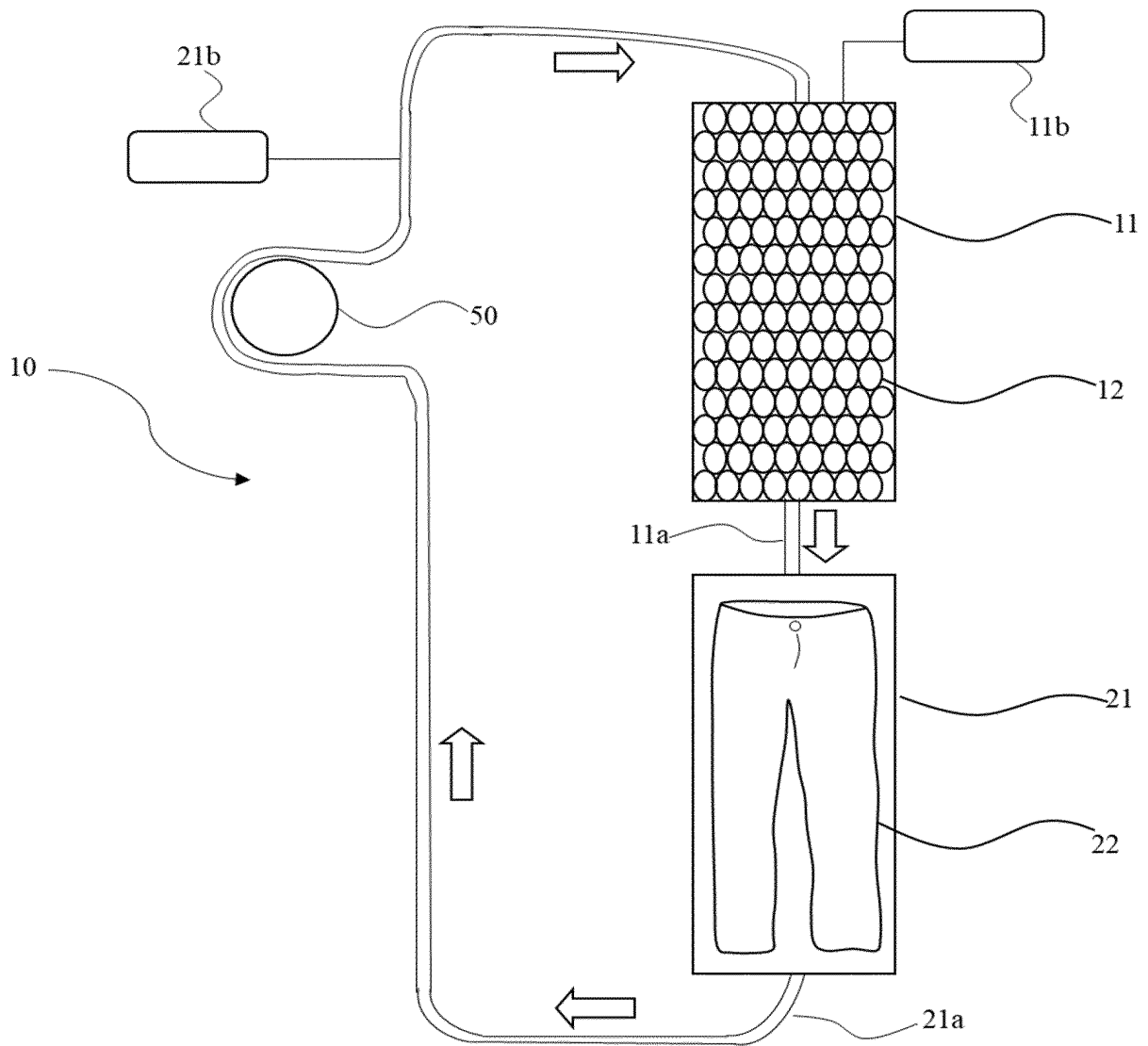


Figure 5

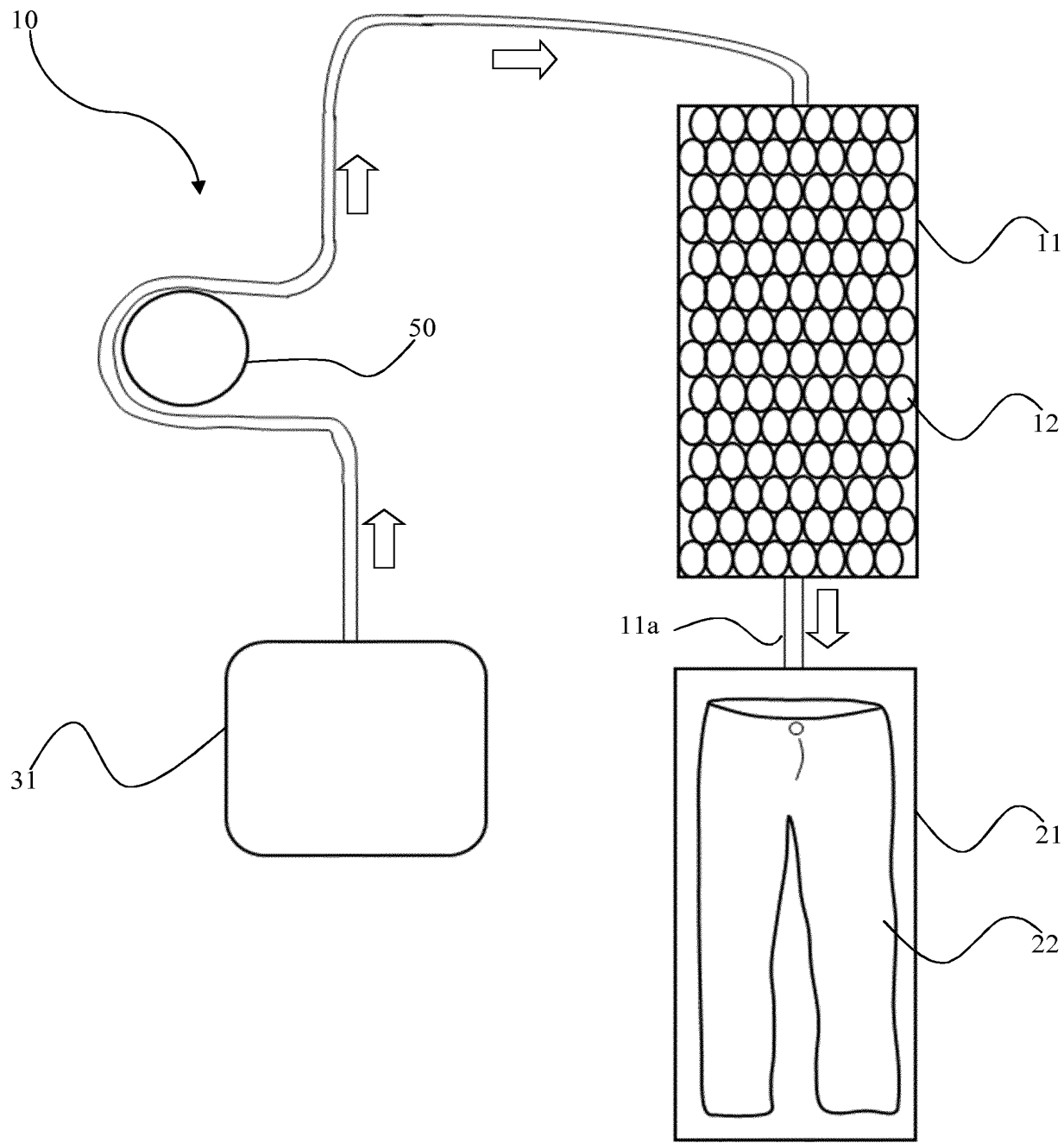


Figure 6

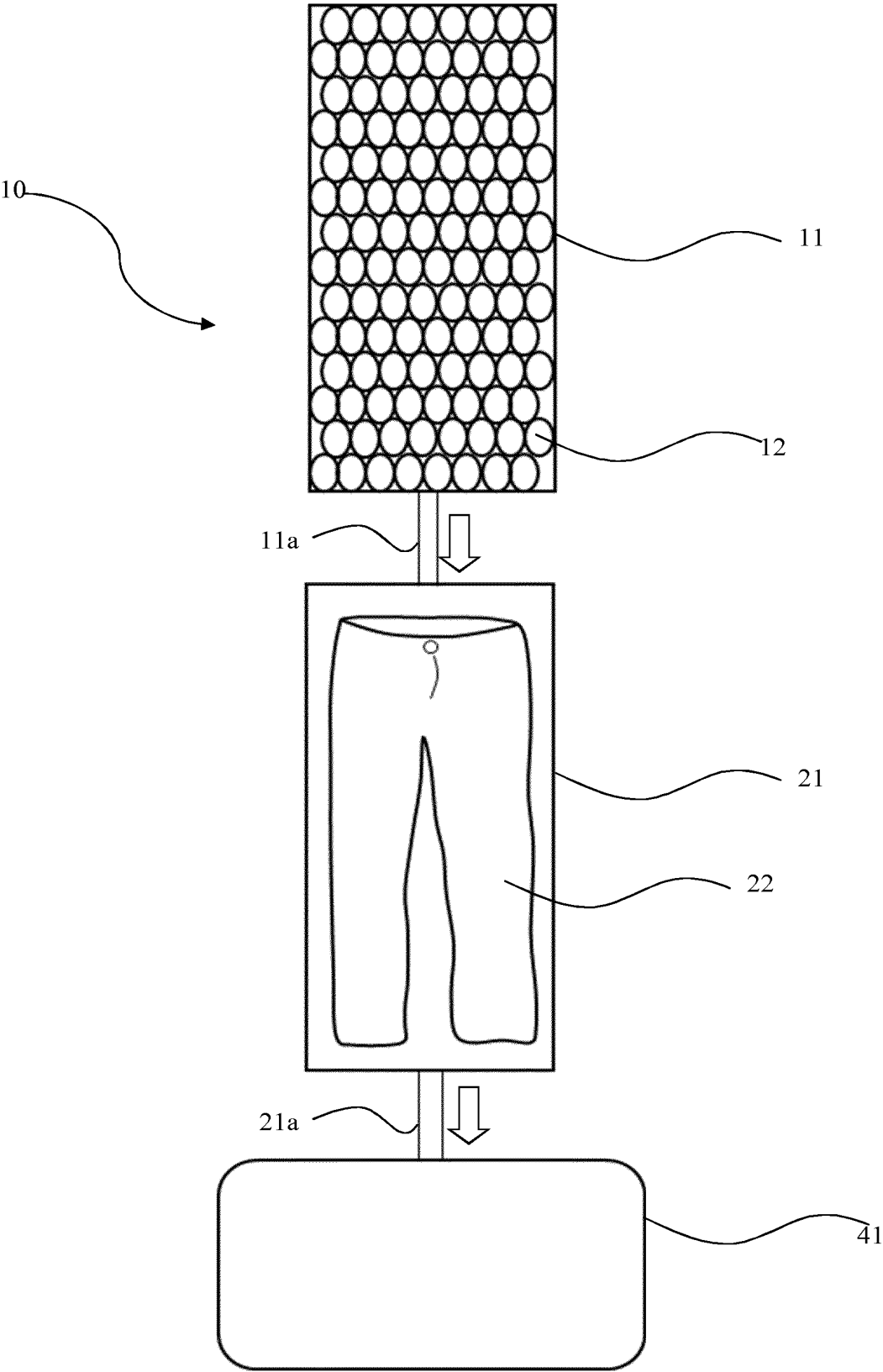


Figure 7

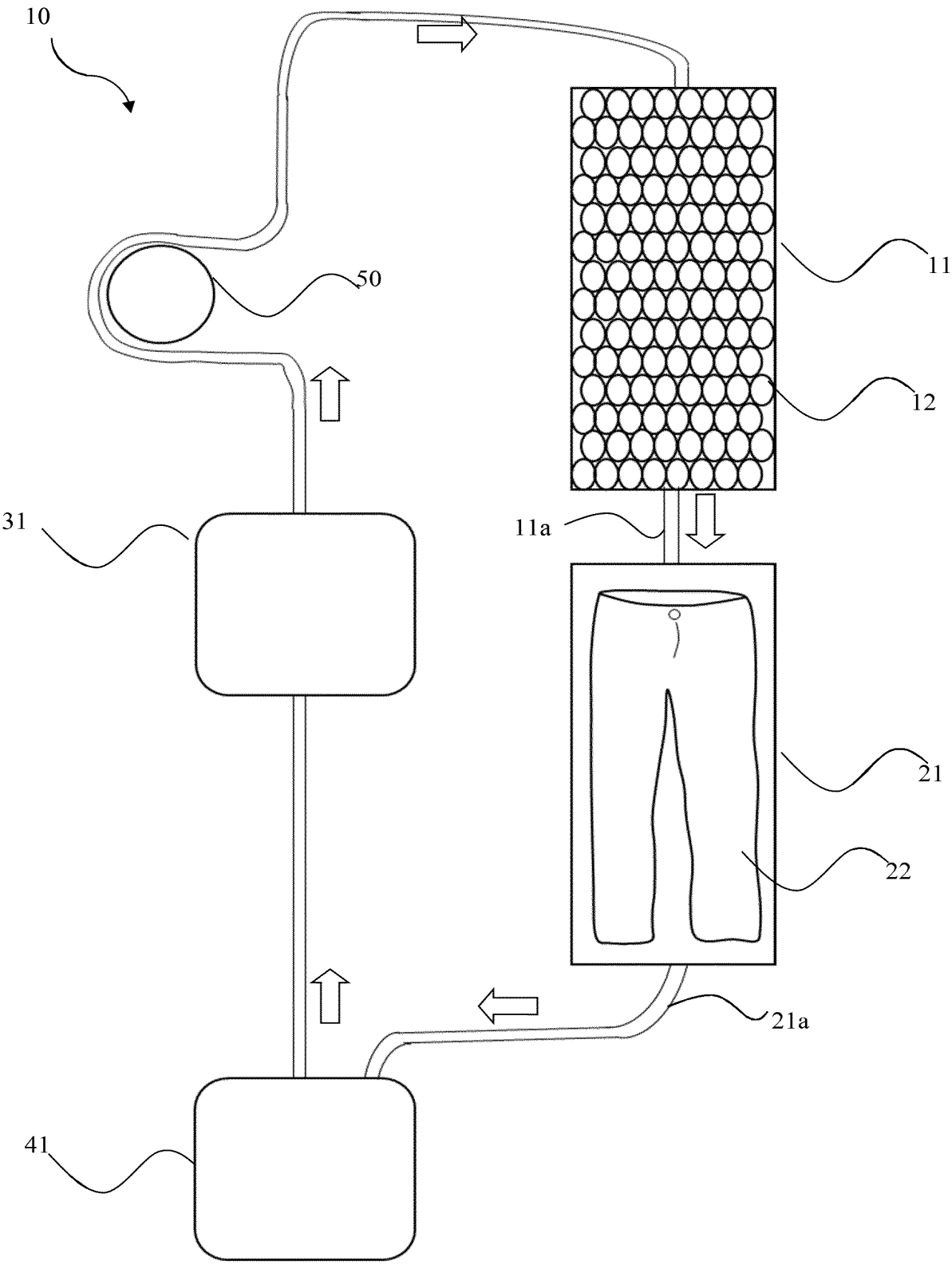


Figure 8



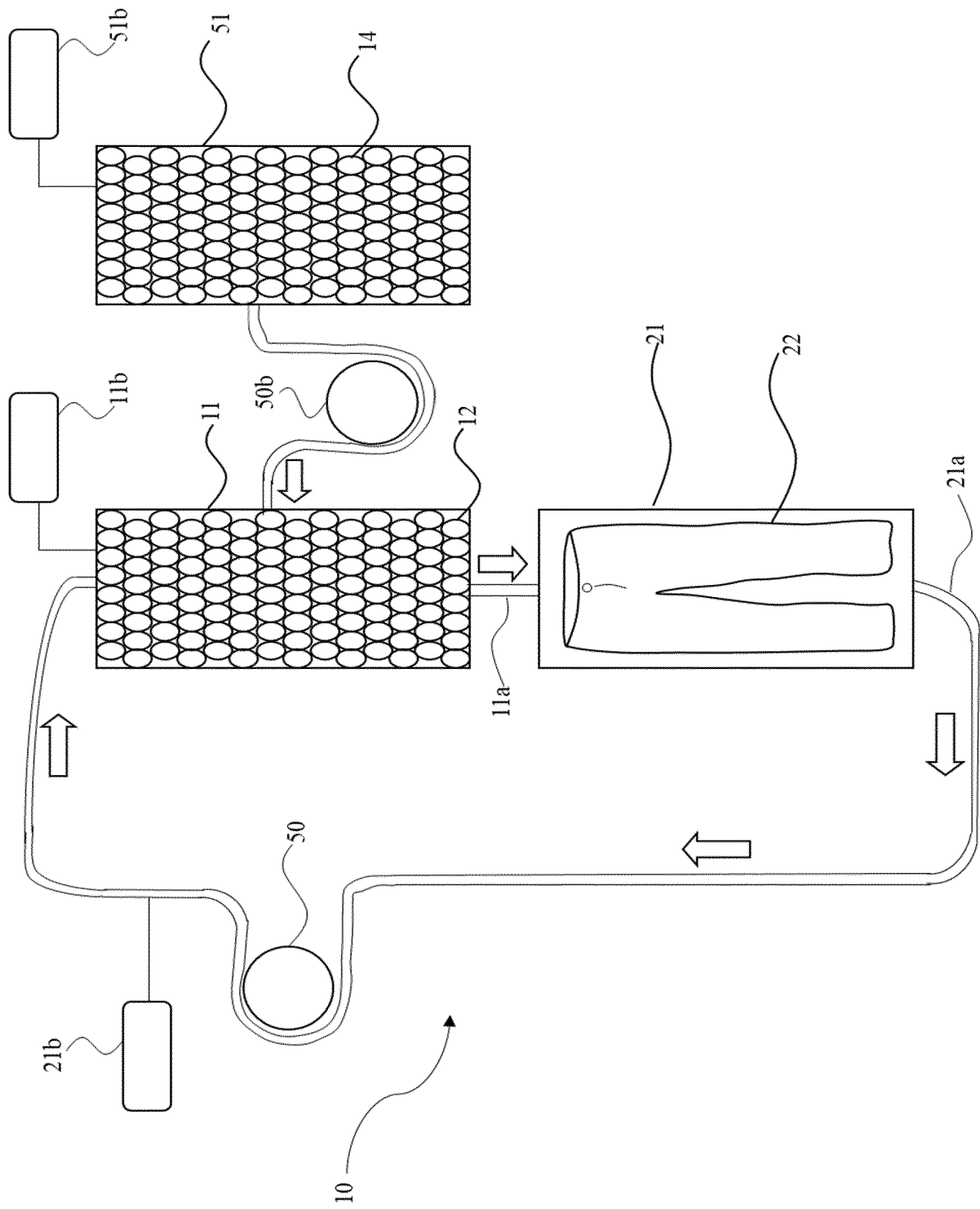


Figure 9

## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/069820

## A. CLASSIFICATION OF SUBJECT MATTER

INV. D06P1/00 D06P1/22 C09B7/00 D06P1/44  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

D06P C09B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 2011 026730 A (UNIV OKAYAMA) 10 February 2011 (2011-02-10)	1-4, 8-11,27
Y	paragraphs [0011] - [0012] paragraphs [0023] - [0024] example 2	4-7, 9-11,23
X	----- Anonymous: "Indigo   Encyclopedia.com", 2016, XP055578266, Retrieved from the Internet: URL:https://www.encyclopedia.com/science-a nd-technology/chemistry/organic-chemistry/ indigo [retrieved on 2019-04-05]	12
Y	The Manufacturing Process: Natural extraction ----- -/-	9-11, 13-16,24



Further documents are listed in the continuation of Box C.



See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

8 April 2019

Date of mailing of the international search report

24/04/2019

Name and mailing address of the ISA/

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## INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2018/069820

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	figure 1	9-11, 13-16
X	----- ANA RIOZ-MARTÍNEZ ET AL: "Exploring the biocatalytic scope of a bacterial flavin-containing monooxygenase", ORGANIC & BIOMOLECULAR CHEMISTRY, vol. 9, no. 5, 2011, page 1337, XP055578275, ISSN: 1477-0520, DOI: 10.1039/c0ob00988a	19,20
Y	Conclusions; page 1339, column second	4-7, 13-16
X	----- EP 0 288 092 A1 (AMGEN INC [US]) 26 October 1988 (1988-10-26)	21,22, 25,26
Y	example 3 column 10, lines 3-18	23,24
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Y	paragraph [0025] example 9	23,24
A	----- WO 2018/002379 A2 (NOVOZYMES AS [DK]) 4 January 2018 (2018-01-04) the whole document	1-27
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Information on patent family members

International application No

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